Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted January – June 2022

Contents

1. The role of tissue-resident immune cells 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

immunology, tumour immunotherapy, cancer, wound healing

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We will investigate the roles of organ-resident immune cells in regulating local inflammation, which is critical in both beneficial processes such as wound repair, or disease states such as fibrosis and cancer 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?

We know that immune cells circulate in the blood, and can enter organs in response to different diseases such as infections and cancer. It is becoming clear that there are certain immune cells that reside as 'tissue-resident' cells in specific organs, although relatively

little is known about how these cells are locally regulated, and what their functions are. Recent findings show that the microenvironment of the organ can instruct how some of these tissue-resident immune cells behave; therefore it is likely that tissue-resident immune cells are tuned to respond to organ-specific challenges. We are specifically interested in a recently discovered tissue-resident immune cell, called group 2 innate lymphoid cells (ILC2). We know that ILC2 play an important role in parasite infection and allergic disease, but still little is known about their roles in cancer or tissue repair. We will build on our expertise in ILC2 immunology, tissue repair and cancer modelling in mice to better understand how ILC2 can locally influence inflammation in these important disease conditions. Ultimately, we hope to control the function of ILC2 to improve anti-tumour immunity or wound repair, which may result in better clinical treatments.

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

We will generate:

A better basic understanding of the regulation and role of tissue-resident immune cells within specific organs.

A better understanding of the roles of tissue-resident immune cells in tissue repair, fibrosis and cancer development.

New reagents that will be shared with the scientific community (i.e. new genetically engineered mice, or cell lines/organoids derived from mice).

Data that will be shared with the scientific community (uploaded to open-access data repository).

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

The following people will benefit:

In the short-term (1-5 years), new basic scientific research will benefit the scientific community.

In the medium-term (>5 years), new information will lead to increased use, or development, of new reagents (benefit industry), or result in translation of pre-clinical research (benefit patients).

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

We will maximise the output by:

Publishing all findings in a timely manner. We only publish in open-access journals to ensure free access to our findings.

We will openly share reagents, data and expertise with collaborators. Valuable reagents will be deposited in open-access repositories, or maintained in easily shareable formats at our Institute. Published datasets will be deposited in open-access repositories. Expertise will be shared via detailed methods publications in open-access journals, or on our website.

We generally include negative findings in our publications.

We disseminate new knowledge related to animal use at local or national animal-users meetings, or scientific conferences.

Species and numbers of animals expected to be used

- Mice: 25000

Predicted harms

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

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

Mice have many similarities with humans in terms of genetics, immunology, and cancer formation. Most current anti-tumour immunotherapies were developed using basic scientific research in mice. Laboratory mice are widely used to study immunology and cancer, and there are many important genetically engineered mouse models available to carefully investigate the function of specific immune cells in health and disease. Similarly, cancer development can be accurately studied using different mouse models.

For tumour studies, we require the use of adult mice because: 1) the tumours we study occur primarily in adults, and 2) we study the interactions of the adult immune system with cancer. For studying the tissue-resident immune system we primarily use adult mice (>95%), although some studies will involve neonatal or juvenile animals where early activation or modulation of the immune system is induced to study the early development of tissue-resident immune cells.

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

We will perform two lines of investigation:

We will investigate the basic biology of tissue-resident immune cells. For this work we will manipulate tissue-resident immune cells to understand how they are turned on/off, and what their function is. We will expose mice to stimulants (i.e. injection of a protein, or exposure of mice to inhaled substance), or administer a test drug to understand if we can block activation. To translate our findings to human disease conditions, we will use models of disease where tissue-resident immune cells play a role (i.e. fibrosis of specific organs, which involves several administrations of substances that reproduce human fibrotic disease). While the majority (80%) of these experiments are short (1-2 weeks), some disease models take longer (several months).

We will investigate how tissue-resident immune cells are involved in cancer. For this work we will combine our methods from the previous section (where we understand how to control tissue- resident immune cells), and apply it to mouse models of cancer. Cancer models generally fall under a) genetically engineered mice that spontaneously develop cancer, or b) mice that are implanted with tumour cells. Regarding the latter, we will inject mice with tumour cells (into the blood, or another relatively painless route), or surgically implant mice with tumour cells into a specific organ (i.e. pancreas). The majority of cancer experiments will last less than 3 months (80%), although some models that accurately mimic tumour formation will take longer (3-18 months).

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

Related to the two lines of investigation:

We will investigate the basic biology of tissue-resident immune cells: These experiments will cause short-term discomfort associated with the administration of reagents (i.e. injection of substance). Some of these experiments will cause short-term (<1 day) discomfort caused by activation of immune cells (i.e. inflammation), which may include mild localised pain or irritation. Mice will be given painkillers in experiments that are known to cause persistent (>1 day) or more serious acute discomfort (i.e. pancreatitis).

We will investigate how tissue-resident immune cells are involved in cancer: These experiments will cause short-term discomfort associated with the implantation of tumour cells (i.e. injection); Surgical implantation of tumour cells will cause medium-term discomfort, that will be managed by painkillers given before and after the surgery. Tumour formation will cause long-term discomfort associated with cancer; this can include:

a) physical hindrance due to tumour mass
b) impaired organ function due to tumours, such as shortness of breath (lung cancer), or gastrointestinal malfunction - leading to weight loss or ascites (pancreatic cancer)
c) pain due to tumour growth. We will carefully monitor mice in these studies, and have specific thresholds to limit adverse effects.

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

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

Some mice will be kept for breeding, or experiments without any procedures performed (sub-threshold) (30%)

Some mice will be exposed to procedures that cause mild adverse effects (40%)

Some mice will be exposed to procedures that cause moderate adverse effects (30%)

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

- Killed
- Used in other projects

Replacement

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

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

Tissue-resident immune cells, and their role in wound repair and cancer development can only be accurately studied in animal models. Mice represent the best model organism, given the similarities between mice and humans.

Major specific reasons for using mice are:

There are many shared reagents available to immunologists and tumour biologists that work for mice, including antibodies for mouse proteins, or genetically altered mouse models that specifically focus on immunology or tumour biology.

There are many disease models available in mice already, which are known to accurately model human disease.

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

We use non-animal methods, or methods that reduce animal use, wherever possible. For example, we generate cell-lines from mouse tissues, which can be used in experiments that don't use mice. We also developed 'organoid' or 'explant' cultures, where one mouse organ is made into many small organs, or organ slices, which can be used for many future experiments (thus essentially replacing mice).

We also use bioinformatic methods to study, and model, immune cells and tumours. For these experiments we still require small numbers of animals to obtain immune and tumour cells; however, these experiments generate very rich datasets, which can be used to predict how cells interact, or react in different diseases. Importantly, these are only predictions that need to be validated in either cell-lines or in mice.

Why were they not suitable?

However, to accurately model the microenvironment of tissue-resident cells, one needs to consider the complex interactions that occur in a live animal. For example, the immune system relies on constant interaction with blood-derived cells, and lymphatic drainage, which cannot be accurately studied outside of the body. Tumour development also behaves very differently inside our bodies compared to cell-lines, which are not exposed to the selective pressures of the tumour-microenvironment.

Therefore, while we use non-animal models wherever possible to address more simple questions, we also need mouse models to study how immune and tumour cells behave within the body.

Reduction

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

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

The majority of the lines in the colony will be maintained on the C57BL/6 mouse strain background which is optimised for immunological studies. This has been used as the basis for our breeding strategies. An assessment of the reproductive performance of the C57BL/6 strain has been detailed in the Laboratory resource: ‘Breeding Strategies for Maintaining Colonies of Laboratory Mice’ produced by The Jackson Laboratory.

As standard, our breeding stock will be maintained at minimal levels with 1-2 breeding pairs of different ages at any one time. Breeding pairs are mated typically at 6-8 weeks old and allowed to have 5-6 litters (approx. 30 week breeding period). Replacement breeders will be taken from the stock mice produced or bought in from commercial breeders (for inbred wildtype strains). We try to avoid sibling mating where possible. During the course of the proposed work we estimate we will breed a combination of approximately 30 mouse strains carrying modified immune genes at any time, split between our Mild and Moderate Breeding Protocols (Protocols 1 and 2). When colonies are expanded for experimental purposes, breeding trios tend to be used to maximise pup numbers of similar age and provide appropriate littermate controls. The majority of mice bred from the expanded colonies will be used for tissue collection for ex vivo work or in subsequent protocols, however there will be the production of unwanted experimental mice which may be used for replacement breeders or culled unused as excess. We currently have 30 mouse lines on each of our mild and moderate breeding protocols - some lines are for breeding purposes only and some lines are expanded for experimental mice. An estimation of the number of breeding females at any one time is given in Table 1.

Table 1: Estimated Breeding Females for Mild and Moderate Breeding Protocols

Mild Breeding (Protocol 1) #	Moderate Breeding (Protocol 2) #
Breeding only (continuation of line/breeder for experimental crosses) 3	Breeding only (continuation of line/breeder for experimental crosses) 1
Experimental crosses 60	Experimental crosses 6
Total breeding females 63	Total breeding females 7

From these numbers, we have estimated the number of mice which will be produced from our breeding strategy over the next five years, taking into account that breeding females will be replaced every 30 weeks (a replacement factor of 1.73 a year). This has given us estimated mice numbers for Protocol 1 19530/5 years and Protocol 2 2170/5 years shown in Table 2, allowing an additional 10% for variations in breeding numbers over the five years.

Table 2: Estimated mouse numbers produced on Mild and Moderate Breeding protocols over 5 years.

	Mild Breeding (Protocol 1)	Moderate Breeding (Protocol 2)
Active breeding females	63	7
Replacement factor (per year)	1.73	1.73
Breeding females per year	108	12
No. of litters perfemale	5.4	5.4
Average litter size	6.09 (born), 5.60 (weaned)	6.09 (born), 5.60 (weaned)
No. pups born per year	3,551	395

No. mice born over 5 years	17,755	1,973
Protocol No. (+10%)	19,530	2,170

For many of our experimental models our initial calculation, in consultation with the establishment statistician, have suggested we require 7-9 mice per group per experiment. Depending on the controls required for individual experiments, this means the use of an average of 20 mice per experiment, 10 mice per group (control and test) allowing an additional 10-20% per group for experimental losses (such as from tumour engraftment problems or technical dosing issues etc). This will be amended as we gain more information from our experiments and will also vary depending on the number of test groups required per experiment (we may, for example, have a control and 2 test groups in some instances). Most of our proposed disease models are well established and robust, however for some cancer studies, for example, there may be a more variable onset and this may necessitate larger cohorts to demonstrate significant differences. Estimated number of experiments to be performed on each experimental protocol are detailed in Table 3 below.

Table 3: Estimated number of experiments and mice used on experimental protocols.

Protocol Number and Name	No. mice per experiment	No. experiments per year	No. mice per year	No. mice over 5 years
Protocol 3 (immune response)	20	50	1000	5000
Protocol 4 (chronic lung inflammation)	20	10	200	1000
Protocol 5 (chronic pancreatitis)	20	10	200	1000
Protocol 6 (subcutaneous tumour)	20	5	100	500
Protocol 7 (intravenous tumour)	20	25	500	2500
Protocol 8 (intraperitoneal tumour)	20	25	500	2500
Protocol 9 (mammary tumour)	20	10	200	1000
Protocol 10 (orthotopic pancreatic tumour)	20	25	500	2500
Protocol 11 (orthotopic lung tumour)	20	10	200	1000

Protocol 12 (orthotopic ovarian tumour)	20	10	200	1000
Protocol 13 (naturally progressing tumour model)	20	10	200	1000

These are our current estimates for the project licence over the next five years, but will be adapted and refined as we increase our understanding of the field and our breeding and experimental requirements.

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

When designing experiments we will initially perform pilot studies to determine the observed effect sizes to ensure that we use the minimum number of mice per group that will be informative. In addition, we will consult with statisticians within the establishment before starting the experiments and throughout when required to ensure statistical confidence.

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

We intend to run the administration of our mouse strains centrally, using the PI and laboratory manager as a central communication point between all users working under the licence and the animal unit staff where the mice are housed. This ensures we can optimise strain breeding to support multiple studies. Should excess mice arise, these can be shared with other researchers who have the authority on their licence to receive them. Collaborating in this way avoids mice being bred and not used, and also avoids duplication of breeding strains within the same facility. The strict control of mouse stock ensures excess breeding is avoided and all mice bred are used as productively as possible. We have quarterly dedicated laboratory meetings to manage breeding strategy to suit the demand.

All mouse lines not in current use are kept on minimal breeding. Where possible, the mice are bred as homozygous lines (providing they do not have a harmful phenotype) and when breeding heterozygotes, littermates are used as controls for experimental work. We have the facility and expertise at the Transgenic Unit of the establishment to freeze embryos/sperm, and this will allow us to store those lines needed for future studies but where no research work is planned in the immediate future.

Every opportunity will be taken to decrease the number of animals used for each experiment, whilst still maintaining the statistical significance of the subsequent data. We would undertake pilot studies when using procedures and models new in our hands, to ensure they are optimised before large scale experiments are carried out. To maximise information, multiple body sites will be examined from each animal and multiple analysis types will be conducted on each sample, where possible. Samples can also be archived, e.g. we have optimised cryopreservation of immune cells and created a bank of peripheral immune cells and bone marrow, which can be retrieved and used for future in vitro functional studies, adoptive transfer for in vivo disease models and for production of

chimeric mice. RNA and protein samples can also be stored, allowing us to revisit a previous study to perform further analysis without the need to repeat the experiment.

We have setup a clinical research study and have optimised protocols to phenotype and analyse various immune functions in various human immune cells from cancer patients, and healthy blood donors. This will reduce our need for some animal models since work can be accomplished in human immune cells obtained from blood and tissues.

Refinement

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

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

Our models and methods fall into five main categories:

Inflammatory models. The majority (80%) of these models target specific organ sites, and cause transient localised inflammation that mimics human disease. Our models are well-characterized in pilot studies, and when appropriate, reagents are titrated to avoid systemic or overt inflammation.

Chronic inflammation models. These models mimic serious human disease (chronic lung inflammation, and chronic pancreatitis) in the majority of mice (75%); we have refined our protocols to cause the least amount of inflammation necessary to model human disease, to limit inflammation to the organ of interest wherever possible, and include prophylactic pain management in models where we anticipate pain.

Tumour metastasis models. These models mimic the spread of cancer to other organs; here we have established specific protocols where the site of metastasis is more predictable. While these models cause suffering associated with tumour spread, the effective targeting of specific organs ensures maximum collection of data per mouse.

Tumour formation models (orthotopic): These models involve the implantation of tumourigenic cells into a specific organ site. While these mice will be exposed to serious harm due to the implantation procedure, these models present the most refined method to study tumour-immune interactions, and are also very robust in terms of disease penetrance. Tumour formation in these mice also follows a more predictable trajectory, and is amenable to more refined monitoring of cancer progression (i.e. bioluminescent imaging).

Tumour formation models (spontaneous): These are the most accurate mouse models of tumour formation. Some experiments require us to look at early stage disease, which is not accurately modelled in the orthotopic models. The majority of these mice (75%) will be humanely killed at an early stage of cancer development, and therefore won't suffer significantly from cancer-related adverse effects.

As we are interested in the role of immune cells, and tissue-resident inflammation in cancer, we will very often (75%) combine tumour models with immune modulation. This may have a protective or detrimental effect on cancer, and we will always perform tumour or inflammatory models independently first to establish a baseline understanding.

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

The diseases or biology related to tissue-specific immune regulation and inflammation apply mainly to the mature, adult, immune system. As these disease or immunological processes occur over the course of several days to months, it is impossible to use terminally anaesthetised animals in most cases. A small aspect (<2%) of our work will involve asking very focussed questions about tissue-specific immune interactions by visualising these interactions in terminally anaesthetised mice.

The processes of tumour metastasis or tumour development are also mainly associated with adults, particularly the cancers we study. As cancer development and metastasis are processes that take time, it is not feasible in most cases to use terminally anaesthetised mice. Nevertheless, we have developed protocols to look at early metastatic seeding in a small number (<2%) of terminally anaesthetised mice.

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

We will use several approaches:

Foremost, we perform pilot studies to define the expected harm; we work closely with animal technical staff to then design the most comprehensive management and monitoring plan.
We communicate very effectively with animal technical staff to capture unexpected adverse phenotypes, or incorporate feedback into our animal protocols. For example, we worked with animal technical staff to improve the non-invasive delivery of post-surgery analgesic and animal housing conditions.
We work with the NVS and other expert users to refine our surgical procedures. For example, our attendance at AWERB and user meetings resulted us identifying a specific expert in orthotopic lung tumour models who shared more refined techniques with us.

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

Unless otherwise specified, the work in this project will be undertaken in accordance with the principles set out in the Guidelines for the Welfare and Use of Animals in Cancer Research: British Journal of Cancer (2010) 102:1555-1577 (referred to subsequently as the 'NCRI Guidelines') and in the LASA Guiding Principles for Preparing for and Undertaking Aseptic surgery (2010).

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

We will use several strategies:

We attend, and present, at dedicated meetings of animal users (i.e. monthly animal users meetings, Animal Welfare Ethical Review Body (AWERB), meetings, national and international scientific conferences). Here we will share and discuss our experiences with

animal experiments, and actively search for more refined methods. We have used this approach to refine some of our existing protocols (i.e. orthotopic implantation of the pancreas and lung), which have led to PPL amendments to incorporate such advances.

We regularly search the literature for more refined protocols, and discuss these with our animal technical staff. We have used this effectively to set up an assay to study early metastatic engraftment of the lung, which was performed under terminal anaesthesia.

We actively engage the animal technical staff, and work closely with technicians, NACWO, and NVS to refine our protocols and are very open to quickly adopting new approaches.

2. Orthopaedic Infection

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

Infection, Implant, Orthopaedics, Trauma, Reconstruction

Animal types	Life stages
Rabbits	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to develop implantable medical devices and device coatings that demonstrate the ability to eliminate any bacteria that adhere to the implant, preventing the development of bacterial infection. The resulting decrease in infection rates and the subsequent human and financial costs would be substantial justifying the use of animals in technology 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?

Infection following repair of broken bones or joint (e.g. hip and knee) replacement is recognised as a significant problem in orthopaedic surgery. Despite the care taken during

surgery bacteria can occasionally invade the implant surface resulting in failure to heal the fracture or new joint being unstable and the need for further surgery to rectify the situation. Current failure rates are up to 2% of initial implantation surgeries (and significantly higher in secondary implant replacement procedures following previous infection), with treatment methods involving removal, prolonged strong antibiotic treatment and eventual replacement of the implant through further surgery. The impact of these infections is wide reaching with significant patient discomfort, increased healthcare costs and in severe cases, amputation of the infected limb.

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

The primary purpose of this project is to address the issue of orthopaedic implant infection, which meets the definition of “control of disease, ill-health or abnormality” from the permissible purposes of the Act. If a technology proves successful in this project and the subsequent clinical trial then it would be coated onto the non-articulating bone contacting/indwelling portions of orthopaedic reconstruction (Hip and Knee replacements) and trauma fixation products producing a significant reduction in the rate of infection associated with these procedures. In time the intention would also be to further disseminate the scientific knowledge gained through peer reviewed publications.

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

This work intends to provide benefits to the patient in terms of reduced morbidity, hospital stays or even mortality. Additionally the technology has the potential to reduce the cost burden to healthcare systems caused by these infections (estimated in the USA to be $1.89bn per year and in UK calculated to be £29.5m per year between 2015 and 2020 , increasing all the time due to the rise of antibiotic resistant organisms and increasing co-morbidities). These benefits should begin to be realised from the point a product utilising the technology is launched, which dependent on development timescales and regulatory requirements such as clinical trials should take place within 5 years of completion of successful animal studies on final product.

The project will also look to benefit the “advancement of biological science” in further development and assessment of the clinical relevance of models for the assessment of antimicrobial coatings for orthopaedic implants. This work will hopefully be published in future, adding to understanding within the literature of orthopaedic device related infections and technologies/strategies that may prevent them. This publication is likely to take place in parallel with product launch, so is likely to take place within 5 years of the completion of successful animal studies on final product.

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

The outputs of the project will be fully exploited through a number of different mechanisms. For example; (a) product support data, (b) pre-clinical regulatory support data, (c) conference abstracts and posters, (d) scientific publications outlining new experimental models of bone repair. Model development may also involve the support of either academics or clinicians, which will assist with knowledge transfer.

Species and numbers of animals expected to be used

- Rabbits: 200

Predicted harms

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

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

Live mammalian vertebrates are required that closely mimic the immune systems and bone repair pathways in humans as much as possible to ensure that any data generated can be translated to the clinical situation. Maturity of the immune system and skeletal maturity are also required for this reason meaning adult animals are required.
The rabbit is a suitable model for the investigation of infection prevention due to their relative susceptibility to infection compared to other small animals (rat, mouse etc.) which allows a more clinically relevant dose of bacteria to be used to generate infection and keeps down the number of animals required to demonstrate significance.

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

Surgically, under general anesthesia with recovery, an entry hole will be made through the tibial crest to the medullary canal of the tibia. The bone marrow is then flushed from canal and the canal is then reamed with a pin of marginally bigger size than the test implant before the canal is flushed for debris. A bacterial suspension is then introduced either prior to implantation of the test pin or in conjunction with this pin. The entry point is then sealed and the soft tissue closed over the site. The estimated time of the surgical procedure is approximately 1 hr. Once the animal has recovered from the effects of general anesthesia it will be carefully monitored by qualified animal husbandry staff until the live phase of the study is completed up to 4 weeks later. At the end of the study, the animals will be humanely euthanised.

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

The project will involve the surgical implantation of a metal pin into a bone canal, which may lead to some degree of discomfort following surgery, although this will be reduced by the use of a minimally invasive technique and the absence of a fracture in the bone which is not required for this work. Any discomfort will be minimised with the use of appropriate pain relief. The establishment of a bacterial infection in the bone of the test animals may lead to some moderate discomfort, however efforts will be made to minimise this with the use of circulating antibiotics and appropriate pain relief. At the end of the studies the animals will be humanely euthanised. In addition any animal showing severe signs of suffering whilst on study (e.g. excessive weight loss, signs of uncontrolled pain, significant lameness) will be humanely euthanised.

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

What are the expected severities and the proportion of animals in each category (per animal type)?
It is expected that greater than 80% of animals will experience moderate harm in work under this protocol. The remaining 20% or less of animals will be classified as non-recovery by failure to recover from anaesthesia due to either adverse reaction to the anaesthetic itself, respiratory failure due to production of bone marrow emboli caused by the nature of the procedure or being euthanised due to irreparable surgical complications.

It is hoped that based on most recent experience with revised anaesthetic and surgical procedures that this will be reduced below 10% of animals.

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

- Killed

Replacement

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

There is no suitable *in vitro* alternative to the animal model for assessment of infection prevention.

However prior to animal testing the basic antimicrobial properties of the coating against the strain of bacteria that we propose to use in *in vivo* studies will be assessed via *in vitro* methods. Bacterial kill away from and at the implant surface will be analysed, ensuring that only once a formulation has shown efficacy (within the limitations of *in vitro* testing) will it be considered for animal studies.

In vitro methodologies, whilst providing a screen to optimise coating formulations, cannot replicate the complexity of device related infection within a host organism. Cellular repair mechanisms, immune defence, inflammation and host animal/microbe interactions cannot be accurately replicated by *in vitro* cell culture/microbiology studies. In order to truly assess the potential of a coating that can reduce implant infection in the human population, *in vivo* studies in the presence of these complex elements, are required. Additionally, prior to granting a licence to carry out the clinical trial and before launch, the final product will require animal testing within the model described in Protocol 1 of this licence application, demonstrating efficacy as detailed in the requirements of “FDA Decisions for Investigational Device Exemption (IDE) Clinical Investigations”, section 8.1 – non-clinical testing, UK Medical Devices Regulations 2002 (SI 2002 No 618, as amended) (UK MDR 2002) the EU Medical Device Directive (93/42/EEC) and EU Medical Devices Regulation (Regulation 2017/745).

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

As stated previously *in vitro* methodologies are used to screen potential test formulations, but these have limitations when it comes to truly assessing the anti-infective efficacy of technologies.

Why were they not suitable?

To date there are no *in vitro* approaches, even those using tissue explants or co-culture of bacteria and immune cells, that can fully replicate the complex interactions of test article, host immune system, circulating antibiotics, inflammation and host animal/microbe interactions. All these elements are required to ensure an accurate replication of the environment a product will be required work in in the clinical setting.

The Project consists of an extensive laboratory based safety and microbiology testing regime to select the most appropriate anti-infective coating for implant surfaces (e.g. an

intermedullary nail), allowing selection of the best formulation(s) for development. While this testing will allow accurate selection of prototypes, it cannot replicate the complex biological interactions at work within the body, thereby making performance testing in animals an essential part of the plan before human trials can commence. Demonstration of efficacy and safety in an animal model is also a requirement of regulatory agencies prior to clearance for human trial and eventual product launch.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 per species required will be determined from a number of sources;

The experiences gained from previous licences.

Advice and approval of protocols by regulatory agencies.

Our statisticians input at the planning stage of the *in vivo* studies to advise on study design, post live phase analysis and to determine the minimum number of animals required to provide sufficient likelihood of a meaningful outcome.

Previous studies or studies reported in the literature will be used to provide variability data to aid this process, or pilot studies will be conducted to generate such data. This will reduce the numbers of animals used in total without compromising the data/information obtained.

Our organisation's AWERB, which will assess all protocols and experimental design prior to the start to ensure a minimum number of animals are used to the meet the study objectives.

Our AWERB review every proposed study in addition to reviewing the protocols proposed in this licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?
Our organization will adopt multiple strategies that will help ensure that the fewest number of animals will be used in the research to address the scientific questions outlined in the project.

SUBJECT VARIABILITY: Variability will also be reduced through the procurement of animals of consistent breed, sex, age and weight ranges and through application of animal acceptance criteria for each study.

BIOSTATISTICS/POWER ANALYSIS: Statisticians will be consulted in the planning stage of the *in vivo* studies to determine the minimum number of animals required to for

statistical analysis and to answer a scientific question being asked. This will reduce the numbers of animals used in total without compromising the data/information obtained. Consultation with a statistician will comprise setting clear study objectives, and ensuring appropriate output measures are collected and analysed using appropriate statistical methods. Sample sizes will be determined based upon the needs of the study which may be tailored for either welfare, pilot, validation or efficacy/non-inferiority/equivalence. Where powered, historical data will be used to determine the appropriate sample size to achieve the required study power. In order to minimise animal numbers used across the project every effort will be made to test as many candidates as possible in a single experiment against a single control group. Based on previous experience with this model the rate of infection/colonisation in control animals is known and so the number of animals in a given study required for a technology to show statistically significant improvement in this rate is also known. This defines the number of animals required per study. This approach, in terms of being an appropriate level of confidence of efficacy, has been provisionally agreed with regulatory agencies.

Where historical information is available the study size will be determined by the minimum numbers required to provide sufficient power (at least 80%) to achieve the desired outcome.

To further minimise numbers, where possible, one sided statistical tests will be used. The objectives dependent on the outcome measures may be to show superiority to a control, non-inferiority to a predicate or gather device performance data. Sources of variability will be controlled by giving careful thought to potential sources of error, bias and variation in measurements, and making every effort made to minimise them. This will include (a) using well-characterised implants that are within specification, (b) defining the success criteria of the study, (c) adopting a consistent surgical technique across the studies, (d) providing adequate time for acclimatization, (e) training of staff, (f) blinding observers and participants to the study hypothesis, and (g) adopting a randomisation schedule in order to reduce bias and interference caused by irrelevant variables.

LOCAL ETHICS (APPROPRIATE EXPERIMENTAL DESIGN): Our organisation's AWERB will assess all protocols and experimental design prior to the start to ensure a minimum number of animals are used to the meet the study objectives. Our AWERB review every proposed study in addition to reviewing the protocols proposed in this licence.

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

IN VITRO TESTING: Prior to studies covered by this project licence extensive laboratory based microbiology testing will be used to ensure that only the most promising technologies will put forward for testing in animals.

QUALITY ANIMALS/VETERINARY CARE/PRE-SCREENING: Health screening of the animals prior to surgery will help screen out individuals that are deemed to be unsuitable for surgery. The loss of animals can also be minimized by providing good post-operative care, use of quality regulated animal suppliers, and planning ahead so that the appropriate number of animals needed for the studies are ordered.

PILOT WELFARE STUDIES: Pilot studies can be used to estimate variability and evaluate procedures and effects. Where the primary output measure of the pilot study is to establish acceptable welfare of animals subject to new procedures under this licence, no more than four animals will be used.

PILOT "POWERING" STUDIES: Where there is no adequate data to power a study, a pilot will be used to gather sufficient data to design a definitive study. Typically, these will be designed to provide a minimum of 10 degrees of freedom to estimate the error. For example, a study with two groups would have a sample size of 6 per group. Where historical information is available the study size will be determined by the minimum numbers required to provide sufficient power (at least 80%) to achieve the desired outcome.

APPROPRIATE USE OF ENDPOINTS - TISSUE SHARING: Where possible, harvested tissues will be recycled for multiple testing, e.g. blood draws, biopsies, CT and histology.

SHARING ANIMALS: For instance, animals euthanized by one investigator can provide tissue for use by another investigator on another licence or protocol.

NEW INSTRUMENTATION AND TECHNIQUES: Using new instrumentation or innovative techniques that can improve precision can reduce the number of animals needed for a study. This has the added benefit of also being a refinement technique for the protocol.

Refinement

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

Which animal models and methods 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 surgical techniques have been refined and our previous experience provides reassurance that no long term lameness/significant pain should result. In addition work carried out under the previous project licences has further refined the surgical technique, anaesthesia and experimental parameters.

Assay methods and *in vitro* testing of test materials for antimicrobial efficacy, biocompatibility, etc. have been developed and used to optimise the materials/factors to be implanted. *In vivo* procedures required to optimally deliver and fix the factor/materials have been fully developed and surgery successfully carried out previously at the our facility. All analysis techniques, including histology have already been fully developed.

Aseptic surgery will be carried out using inhalation anaesthesia. The animals will be sedated using premedication prior to inhalation anaesthesia (e.g. Alfaxalone) with analgesia (e.g. Meloxicam). Analgesia (e.g. Buprenorphine and NSAIDs) will be given post-surgery for a minimum of 24 hours. Previous work has shown no contraindications of the probable analgesics of choice.

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

Live mammalian vertebrates are required that closely mimic the immune systems and bone repair pathways in humans as much as possible to ensure that any data generated

can be translated to the clinical situation. Maturity of the immune system and skeletal maturity are also required for this reason meaning adult animals are required.

The rabbit is a suitable model for the investigation of infection prevention due to their relative susceptibility to infection compared to other small animals (rat, mouse etc.) which allows a more clinically relevant dose of bacteria to be used to generate infection and keeps down the number of animals required to demonstrate significance.

Time (days to weeks) is required for the development of implant colonisation/infection in order to differentiate between successful test technologies and the negative control group therefore recovery from surgery and a live phase are necessary.

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

Refinement applies to all aspects of animal use, from housing and husbandry to the scientific procedures performed on them. Continued investigation into animal refinement will be sought through several sources, e.g. (a) careful choice of animal model, (b) adoption of a multi-disciplinary team with expertise in animal husbandry, housing and care, veterinary science, pain management, engineering and project management, (c) improvements in animal procurement, transportation and quarantine, (d) improvements in animal husbandry such as training of animals and group housing to habituate animals to study procedures to minimise any distress, (e) implementation of housing, e.g. micro- and macroenvironment, (f) increased monitoring and surveillance, (g) refinement in surgical techniques, e.g. minimal invasive surgery that minimize animal pain and distress, (h) appropriate anaesthesia, analgesia and sedatives to minimise pain and (i) post-operative care/recovery and (j) pain management (anesthesia, analgesia).

SOPs will also be regularly updated and documented within our Quality Management System, which is accredited to ISO9001. Staff training will also be made available through attending courses and conferences and integrating with key opinion leaders.

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

Best practice approaches will be used to enhance animal well-being, minimize or avoid pain and distress, and reduce the number of animals required to obtain the desired research objectives.

Best practice on animal care and husbandry will also be achieved through several sources including

(a) our facility's Animal Welfare and Ethical Review Body (AWERB) with an advisory function on ethical matters,

(b) UK Home Office guidelines on Animal Testing and Research https://www.gov.uk/guidance/research-and-testing-using-animals,

(c) NC3Rs, which is a UK-based scientific organisation dedicated to replacing, refining and reducing the use of animals in research and testing (the 3Rs) https://www.nc3rs.org.uk and

(d) consultation of the Guide for the Care and Use of Laboratory Animals (Source: National Research Council of the National Academy of Sciences 2011).

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

The subject matter experts that are employed within the animal facility will engage in continuous professional development that will ensure best practices in pharmacology, radiography, animal husbandry and welfare are regularly adopted during the lifetime of the project.

3. The mechanisms of modulating nerve cells networks and their implications for neuronal excitability

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Autism, nervous system, development, excitability, animal models

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

This study aims to determine how specialized regions of nerve cells control overall brain excitability. We will elucidate the molecular mechanisms that governs the structural modifications of these specialized regions and how such modifications lead to altered nerve cell excitability under normal circumstances and in autism spectrum disorders.

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

Why is it important to undertake this work?

The numerous nerve cells in the brain termed “neurons” modulate the excitability of the brain. This modulation of brain excitability is important for brain development, learning, memory formation, and social interaction. There is growing evidence to suggest that the excitability of neurons may also be regulated by a distinct region of the neuron - the axon initial segment (AIS). The AIS is a dense network of several proteins including ion channels and membrane proteins. Importantly, several autism spectrum disorder susceptibility genes

encode AIS proteins. The structural modifications to the AIS have been reported in several mouse models of autism such as Fragile X and Angelman syndrome.

Hence, the underlying mechanisms of AIS alterations and how it influences neuronal excitability are key questions relevant to the function of neural networks in health and autism. This may eventually offer possible targets for influencing excitability-associated behaviour such as repetitive and restrictive behaviour in patients with autism.

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

The overall research objective is to pursue a detailed analysis of mechanisms of activity-dependent changes to the AIS. This ability to activity-dependent change is called 'plasticity'. Our research will generate considerable data on protein localisation, transport and how these influence AIS plasticity to regulate neuronal excitability. This will be critical for identifying new targets to manipulate neuronal excitability in physiological and pathological conditions.

The novel findings will be published through primary research publications that are available to the wider academic community.

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

The people who will primarily benefit from this research are neuroscientists, cell biologists, developmental biologists, and students. The structural organization and function of the AIS regulating neuronal excitability are fundamental research questions. This study on activity-dependent changes in the AIS will benefit researchers investigating mechanisms of neuronal development, functions, and excitability. Further, this work may reveal how other specialized regions of neurons are assembled and regulate neuronal excitability. This will benefit all researchers who are studying the development of the nervous system and neuronal excitability-related disorders such as intellectual disability, epilepsy, and attention deficit hyperactivity.

The larger community including cell biologists and those who are studying protein transport will be interested in the mechanisms of protein transport and targeting of proteins to specific locations. Disturbances to protein transport have been implicated in several diseases, including Alzheimer's disease and multiple sclerosis. Therefore, my research on how neuronal activity modulates protein transport and facilitates protein targeting can boost that area of research.

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

I will have several collaborations within and outside the institution. This will help in efficiently establishing live-cell imaging and further analysis. I will attend conferences both international and within the UK/EU to share and discuss my research with the wider scientific community, including neuroscientists, cell biologists, and developmental biologists. Our centre regularly publishes articles about the research activities of the centre. I plan to write articles about my scientific research to reach a wider audience of students, scholars, and other institute members interested in research activities. Further, I will participate in science festivals and the Open Day to engage with the general public. I will disseminate our ideas and research to generate enthusiasm about scientific research. Finally, I plan to have regular updates/newsletters about my research activities on my personalized laboratory webpage and on social media platforms to engage with the wider research community, students, and the general public across the world.

Species and numbers of animals expected to be used

- Mice: 2000
- Rats: 750

Predicted harms

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

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

However, to understand anatomical changes of the brain, we need to have a model with a sufficiently developed network of nerve cells. Currently, the mouse and rats are the only mammals where it is feasible and practical to perform the genetic manipulations required for our analysis. Postnatal days (0-
2) days can generate excellent neurons that can grow in a dish. For experiments with brain slices, animals at an appropriate age are chosen. At this stage, the animals will have mature nerve cells and developed networks of nerve cells. This will be important to determine how activity can modulate specialized regions of the brain cells.

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

In order to grow neuronal cells in a dish, animals aged postnatal days 0-2 will be decapitated and brain tissue will be used.

The animals will be humanely killed by an overdose of anaesthetics. Following that, brain slices will be generated for the study.

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

The animals will be humanely killed rapidly to avoid any adverse effects. The genetically altered animals used in this project should not develop any conditions that will cause them any significant harms. They will be bred and maintained at the facility under standard protocols. The animals exhibiting any unexpected harmful abnormal phenotypes will be humanely killed. In the cases of individual animals of particular scientific interest, we will promptly seek advice from the Home Office. At the end of each experiment or at the end of their breeding life, animals will be humanely killed.

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

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

100% of the animals (both genetically altered and wild-type (i.e. normal)) used on this project should experience only a very low level of harm.

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

- Killed
- Used in other projects

Replacement

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

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

In order to understand behavioural/anatomical changes resulting in neuronal excitability, we need to have a model with a sufficiently developed network of nerve cells. Currently, the mouse is the only mammal where it is feasible and practical to perform the genetic manipulations required for our analysis.

Whilst elements of neurodegeneration and neurodevelopment can be modelled in cell culture (growing brain cells in a dish), complex intercellular interactions in the nervous systems must always be referenced to the processes as they occur in either the intact animal or in tissue culture taken from animals, in the case of this project, mice, and rats. The use of live animals is required to achieve the scientific aims. Only mammals have a sufficiently developed nervous system to readily compare to humans. Nevertheless, we aim to make extensive use of cell culture and brain slices recapitulating neuronal development from postnatal mice and rats aged P0-10. These will be ideal for live imaging.

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

We take advantage of several non-animal alternatives and routinely use immortal cells and computer- assisted analysis that do not involve the use of animals.

Why were they not suitable?

The intact nervous system is a prerequisite to study the general excitability of the neurons and hence non-animal alternatives are not suitable 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?
The experimental designs and methods of analysis have been discussed with statisticians and fellow collaborators. We will use the least number of animals to generate scientifically valid data, generally based on the power calculation and existing published literature.

We will generate neuronal cells from the postnatal animals to grow them in a dish for the investigations. This will reduce the number of animals significantly.

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

Generally speaking, statistical analyses generally by 2-tail Student's t-test or one-way ANOVA require no more than 4-5 animals per condition totalling 8-15 animals per experiment because, fortunately, the effects tend to be so clear cut.

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 rat and mice pups to generate our cell culture and slice culture models. This means we can use fewer animals as the breeding pair can be reused for the experiment.

In order to seed 4 imaging chambers with neuronal cells, 2 mouse pups and 1 rat pup are required. However, these chambers can be repetitively imaged for up to 4 weeks. This is extremely efficient and means we do not have to keep generating new neuronal cell culture to study longitudinal changes with time in neuronal development.

Refinement

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

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

We are using genetically altered mice to understand the function of genes that regulate fast communication of the nervous system network. The genetically altered mice will be maintained by breeding with wild-type mice. They will also be bred for analysis by generating mice where the activity of genes will be removed.

For cell to cell communication in a developmental context, such as in neuronal development, it is essential to look at in vivo biology. The most efficient and most effective way of assessing the function of proteins in this context is to either increase or remove the activity of their genes using genetic alteration techniques.

In general, the severity level of our genetically altered mice is Mild. It should also be noted that none of the genetically altered animals where the activity of the gene of interest is either removed or increased has adverse phenotypes. We will use wild-type rats (e.g Sprague Dawley) at postnatal days P0-10 to generate our slice culture and cell culture systems using brain tissue. These are excellent model systems to study neuronal excitability.

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

Postnatal days (0-2) days can generate excellent brain cells that can grow easily in a dish. For slice culture, animals at an appropriate age are chosen. At this stage, the animals will

have mature nerve cells and a developed network. This will be important to determine how neuronal network activity can modulate the specialized regions ofthe nerve cells.

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

The severity limit is mild, and only competent and highly trained staff will conduct the experiments. We take advice from the local veterinary team in the facility to minimize welfare concerns.

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

To improve the welfare of the animals, anaesthesia, analgesia and general protection will be provided to the mice to avoid any suffering prior to manipulation or killing for the experimental procedures, using approved methods.

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

We regularly review the scientific literature and consult veterinarians to alleviate adverse effects. We constantly have discussions with colleagues and attend scientific seminars to stay informed about advances in the 3Rs.

4. Pathobiology and treatment of brain tumours

Project duration

5 years 0 months

Project purpose

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

Key words

Brain Tumour, Nanoparticle, glioblastoma, cancer therapy, CRISPR Cas9

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?

In this study we generate brain tumours in mouse models, which are engineered to be as similar as possible to malignant human brain tumours. These human tumours, glioblastoma and astrocytoma, are still difficult to treat, and we will use the mouse model to find novel ways of getting treatment drugs into the brain tumour.

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

Why is it important to undertake this work?

Over the last 10 years much has been learned about how human brain tumours can be diagnosed more reliably. This was possible through genetic studies which have identified tumour-related genes that are unique to certain types of glioma. We are re-creating these tumours in mice to find better ways to treat these tumours. To do this, we need to find novel drugs, and we need to bring them in small vehicles (so- called Nanoparticle) into the

tumour cells. This method is too early to be tested in humans and we first need to find out what works well and what is unsafe, using our models

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

At the end of this project we will have a better model for a certain type of human astrocytomas, a brain tumour that grows diffusely into the brain and is difficult to control. We will also have tried novel treatments using encapsulated drugs that we can deliver to these brain tumours without causing too much harm to the healthy parts of the body.

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

The primary beneficiary will be the scientific community, including basic scientists, oncologists and members of the medical community involved in clinical trials. Even though it sounds very ambitious, it may be possible that there are early clinical trials at the end of this project (i.e. in 5 years) using these nanoparticles. Medical safety tests for these nanoparticles have already been passed, and in this project we want to use the nanoparticles to deliver certain drugs to the tumour and test how effective this method is.

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

We have an established track record in publicising our work on national and international conferences, and of course through peer reviewed publications. Through our work with brain tumour charities, we can also communicate such results with charities, who often have a wide network of patient interest groups.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

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

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

The mouse is a well characterized model system to mimic human diseases. We have chosen to use a genetic approach to study brain tumour formation, as it allows for controlled and predictable experimental conditions as compared for example to carcinogen-induced mutagenesis, which has been often used in the past.

To mimic the human brain tumours arising in young adults (astrocytoma) we need to mimic the time at which these tumours start growing, also in mice. Therefore, the induction of tumours happens in very young mice, and the tumours become mature in middle aged mice (60-80 days of age).

Typically, what will be done to an animal used in your project?
The first step to create animals of the desired genetic make up, is the breeding of several strains of genetically modified animals. These animals are basically healthy and show no adverse effects. The experiment starts with an injection of very small volumes (1 µL) of a

solution containing a virus which causes genetic changes in specific brain cells. This leads to the growth of these brain cells into a tumour.

Typically, such tumours are induced in newborn mice and tumours develop after 30 -60 days, depending on the combination of genes. Once these tumours grow, we inject nanoparticles, which are harmless, biodegradable substances. However, these nanoparticles can contain treatment drugs which are designed to reach the brain tumour. Whilst this is happening, animals may be monitored by imaging methods, to show the growth or shrinkage of these brain tumours.

At the end of an experiment, the animal will be humanely killed (schedule 1 method) and cells will be derived to be grown in the laboratory, or brains will be analysed using histological and immunohistochemical methods.

Number of procedures: breeding, intracerebral injection (tumour induction), intraperitoneal injection on multiple occasions (delivery of nanoparticles), and schedule 1 killing. Optional additional procedures are imaging and behavioural tests

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

The main aim of this project is to understand the growth of brain tumours in a mouse model. Therefore, our experiments need to form cancer cells in the brain, which eventually will grow into tumours. A Brain tumour has a side effect on the behaviour of these animals. These include weight loss, reduced movements, and withdrawal from the group. Therefore, our trained animal staff monitor these animals on a regular basis (daily) and specifically watch out for these signs. Once a mouse shows one or more of these signs such a sign, it will be humanely killed and used for further analysis, which includes a thorough tumour analysis with molecular biology methods, growing cells from it or the analysis by a pathologist. The expected level of severity in the above circumstances is moderate.

A proportion of the animals will be treated with nanoparticles, a method to deliver cancer drugs to brain tumours. In these experiments we will establish if the drug has any tumour delaying effect. These experiments are devised as follows: (i) treatment and control cohorts are culled at the same time, as soon as one of the cohorts shows clinical signs. This is to establish by histological analysis if the treatment group has a smaller tumour size. (ii) each of the cohorts (treatment/therapy and control) will be culled when neurological signs appear, to establish the survival differences between control and treatment group.

Imaging experiments on the treatment and control cohort will be performed before the expected onset of symptoms. These timelines will be established prior to planning the imaging experiments.

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

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

Production of genetically altered mice: severity: moderate (5%), all other animals mild severity.

Neural development and neoplasia: severity moderate (80%), all other animals mild severity

Superovulation: severity mild (all animals)

Embryo recipients: severity moderate (20%), all other animals mild severity

Generation of founders: severity mild (all animals)

Vasectomy: severity moderate (all animals)

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

- Killed

Replacement

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

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

The main aim of this project is to understand the growth of brain tumours in a model that can be genetically modified but also has sufficient similarities to the human brain to be realistic. To study the growth of a tumour, we need a model in which the tumour growth can be followed with methods similar to those used for human brain tumours, such as magnetic resonance imaging.

Complex diseases such as CNS development and tumourigenesis can only be studied in whole organisms and the mouse is a commonly used and well characterised model system to mimic human diseases. The generation of new mouse models, closely mimicking the human neoplasms, are essential models to understand the cell of origin and pathogenesis of brain tumours, study the direct interaction with the central nervous system, and evaluate how the tumour response to therapeutic approaches

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

We are already using alternatives to *in vivo* models. Often, some hypotheses are generated by cells or cell lines that are derived from such mice, or indeed from human tumours. Once we have identified biomarkers identified in vitro, we validate them by inducing or repressing genes in our *in vivo* models.

We have successfully replaced some of the *in vivo* experiments with organoids, which can be grown from tumour stem cells, in a cell culture dish. Other methods use organoids derived from human brain cells and tumour cells interact with the non-neoplastic organoids. However, the treatment of a brain tumour with a drug, delivered via nanoparticles, can only be simulated in a live organism. This live organism must have developed a tumour that is sufficiently similar to human tumours and therefore the organism must be amenable to genetic manipulation.

Why were they not suitable?

The use of three-dimensional organoids is very good to study some basic behaviours of tumour cells, such as clonal expansion and tumour cell diversity (in fact we found that for studying these features, in vitro approaches are superior to animals). However, the in vitro (i.e. in a cell culture dish) cells or organoids cannot replace a live system in which nanoparticles are transported through the body to reach the brain. Also, such in vitro models cannot replicate the appearances and growth of human tumour, in particular the interactions with the surrounding normal brain.

Reduction

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

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

The numbers of animals were estimated based on previous experimental data, efficacy and efficiency that we established with previously tested methodologies.

This number needs to be supplemented by a contingency to allow for testing experimental variables, new drugs that become available during the course of the project, and to account for additional genes that need to be examined in the mouse models.

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

By using live animal imaging (for example MRI, magnetic resonance imaging), we can follow the growth of tumours at various time points in development without the need of killing these mice, thus reducing the number of mice.

We derive stem cells from genetically modified mice, which can then be further studied in cell culture. This has already the previous license reduced the number of mice that would have undergone an invasive procedure. The benefit of this approach is the generation of differential expression data, which will subsequently be evaluated and validated *in vivo*.

We have determined that 12 mice are needed per group in our xenograft studies (transplantation of human cells into mice) in order to achieve statistically significant and reproducible results.

We constantly evaluate our methodologies and aim at best practices in relation to the 3Rs. For example, we found that by injecting a new type of virus into newborn mice significantly speed up the development of the tumour. Previously it took nearly one year to form a tumour, and now it is just above a month. Also, the rate of tumour formation is now nearly 90%, in comparison with 20% previously. This considerably saves on mouse numbers and on cost.

We have replaced the need for complex breeding schemes by a faster, simpler gene modification using the CRISPR Cas9 method

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

We are using an optimised model system allowing for an assessment of an experimental outcome after only 4-6 weeks. This allows a quick modification of experimental variables where appropriate. The optimisation is the use of a highly effective gene delivery system to the brain to induce tumours, and a genetic construct that generate brain tumours at almost 100% efficacy. Any modification of a system reducing the efficacy will be identified within very short periods of time.

The xenografting model uses established values for efficacy of tumour growth and this allows us to adapt the number of recipient animals accordingly, and with the aim to minimise overall experimental animals.

Finally, our overall genetic approach to induce tumours leads to a substantial reduction of experimental animals, by obviating the need of many breedings and intercrossing to generate desired genotypes.
Many of our tumours are now induced using the CRISPR Cas9 method, allowing targeted and specific tumour induction, with substantially reduced numbers of animals with unwanted genotypes

Refinement

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

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

The mouse is a well characterized model system to mimic human diseases. We have chosen to use a genetic approach to study brain tumour formation, as it allows for controlled and predictable experimental conditions as compared for example to carcinogen-induced mutagenesis, which has been often used in the past. The 2nd approach is the use of mice receiving patient-derived xenografts, which is a worldwide accepted, and standardised procedure. Such standardised procedures are important for the comparability of data and eventually for publication.
Why can’t you use animals that are less sentient?

We are indeed using newborn animals for nearly all inductions of intrinsic brain tumours. This is a significant change from previously used protocols where 6-week-old mice were used. For the injection of patient-derived glioma initiating cells (“xenografts”) we have however to use an internationally accepted and validated standard method, to be able to compare our data with those generated by other groups.

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

The procedures are regularly reviewed by the named veterinary surgeon and the NACWO. Also our team continuously strives to minimise harm by refining procedures, establishing safer anaesthetic methods, and using effective (daily) surveillance of all experimental animals, we avoid gaps during which animals could develop neurological signs caused by brain tumours.

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

We are following institutional guidelines and we follow the recommendation of the AWERB. The application has been formally peer-reviewed, and follows guidance published in RSPCA, the NC3Rs' ARRIVE Guidelines 2.0, and LASA, 2015, Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies. A report by the RSPCA Research Animals Department and LASA Education, Training and Ethics Section. (M. Jennings ed.)

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

The project licence holder and all members of the team with personal licences participate in monthly animal committee meetings. During these meetings such advances are being discussed, encouraged, and continuous review of practice ensures we are up to date with the 3R's, and this is documented in summaries, actions points and minutes of these meetings. 3R's are also encouraged by funders of the project, and any reduction will also have beneficial financial impact on the project.

We also receive NC3R newsletters, and are part of a department-wide incentive scheme to reward individual members of staff for improving the use of the 3Rs in their work. I encourage my staff members to attend NC3R talks and trial any new methods that would improve our adherence to the 3Rs.

5. The impact of integrin a1b1 signalling on polycystic kidney disease

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

Kidney, ADPKD, integrin, Therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To determine if the integrin a1b1 receptor is required for cell proliferation in a mouse model of Autosomal dominant polycystic kidney disease (ADPKD)

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

Why is it important to undertake this work?

Autosomal dominant polycystic kidney disease (ADPKD) is the most common, potentially life threatening hereditary disorder, affecting 1 in 1000 individuals in the UK and around the world. There is currently no cure for ADPKD and new treatments are required to relieve the burden placed on the NHS and other healthcare systems worldwide.

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

The major output from this project will be new knowledge regarding the importance of a molecule, integrin a1b1 (introduced below), in cyst formation.

Cysts are fluid-filled sacs that are a major feature of ADPKD and prevent the kidney from performing its vital function of filtering toxins out of the blood. My previous findings implicate a role for integrin receptors in the formation of cysts. Integrins are receptors on the surface of cells that interact with proteins outside of the cell. These interactions influence many diverse processes inside the cell (and also allow the cell to influence the environment outside of the cell). There are 24 different types of integrin receptor, they are composed of one alpha subunit and one beta subunit. The integrin receptor that I am interested in is the alpha 1 beta 1 (a1b1) receptor as its expression is much higher in zebrafish and mouse models of ADPKD and in human ADPKD patient tissue. Investigating the role of integrin a1b1 further, in a physiologically relevant animal model of ADPKD, will rigorously test if targeting this receptor is a viable future therapeutic option to prevent cysts forming in the kidney.

The findings of this work will be disseminated in peer-reviewed publications, to scientific audiences at conference meetings, and to the lay public via forums such as Twitter and presentations/conversations at events held by the UK PKD charity. Should integrin a1b1 be (as predicted) found to be important in cyst formation, a new target for therapeutic treatment of ADPKD will be revealed.

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

In the short-term (3-5 years), the scientific research community (in particular researchers involved in cystic kidney diseases) will benefit from the outputs of the project. New targets for therapies to treat cystic kidney diseases (such as ADPKD) will be revealed, thus in the mid- to long-term (5-15 years) these targets will be able to be further tested with the aim to develop them for clinical application that will benefit patients with ADPKD and potentially other cystic kidney diseases.

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

All data accrued in the project will be disseminated regardless of the outcome. This will primarily be in the form of scientific publication (preferably in high-impact open-access journals). To carry out the project I have already developed collaborations with world-leading researchers in the USA and Europe, and I will use their guidance and expert input to maximise the outputs from the work. The results of the work will also be of keen interest to other world-leading experts and I will interact with these individuals in the hope of fostering new collaborations and to ensure the results are disseminated to the widest audience. I am also passionate about disseminating the work to lay audiences, in particular to patient groups. To this end, I will present my findings to attendees at workshops setup by the UK PKD charity.

Species and numbers of animals expected to be used

- Mice: Up to 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 mouse model is the most rigorous system to study ADPKD and is the most comparable to the human disease. The mouse model of ADPKD that will be used develops cysts in its kidneys in the first 4-weeks of life, and so this life stage will be used to carry out most of the investigations. A small proportion of animals will be grown to 6 months of age to determine the longevity of protection that integrin a1b1 depletion offers in the mouse ADPKD model. 6 months of age has been chosen as it is prior to the onset of kidney failure and liver damage that occurs in the ADPKD mouse model. This older time-point will permit comparative analysis of the effects that the presence or absence of integrin a1b1 has in ADPKD mice over a longer lifespan.

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

The mice will be bred and maintained using standard protocols. ADPKD mice will be crossed to a mouse strain that is depleted of integrin alpha 1. All mice to be used for data gathering will be killed using Schedule 1 methods at the desired life stage. Mice will also be genotyped, which will most likely be done by ear biopsy, or by sampling either the blood or hair, or by mouth swabbing.

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

The ADPKD mice may experience kidney failure, liver damage, abdominal discomfort, kidney infection, and rapid weight loss due to cyst formation in the kidneys. These adverse effects are unlikely to occur as the ADPKD mice will be humanely killed at either 4-weeks or 6-months of age, prior to their onset.

The mice that are singly-deficient in integrin alpha 1 experience no adverse impacts and/or effects. The crossing of ADPKD mice to mice deficient in integrin alpha 1 is predicted to reduce the adverse effects present in the ADPKD mice, primarily by reducing cyst size and maintaining kidney function.

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

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

All strains that contain the ADPKD genotype will be classed as moderate severity due to the potential that these mice have the adverse effects described above. To reduce harmful effects, I will be mainly focussing my research on an early stage of life (4-weeks of age). At this time-point, the mice have developed cysts but have limited adverse effects as a consequence of their genotype. If ADPKD mice at 4-weeks of age show improvement in their symptoms as a consequence of deletion of integrin alpha 1, I will grow some mice to 6 months of age to test the longevity of protection that loss of integrin alpha 1 provides. Even at this stage of life, kidney failure will not have begun and other symptoms that are characteristic of ADPKD are not predicted to be experienced at 6-months of age. However, the ADPKD mouse model I am using can cause harmful effects, including abdominal discomfort, kidney infection, kidney failure, and other non-kidney abnormalities (such as liver and bile duct damage). This is why I am denoting the severities of the mice as moderate even though the life-stages I will use will minimise the number of mice that experience moderate severities.

The Integrin alpha 1 depleted mice are a mild severity.

For the experiments, inheritance is Mendelian and so a quarter of the mice used will have the adverse effects associated with the ADPKD mouse strain.

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 integrin a1b1 receptor interacts with molecules in the outside of the cell that make up the extracellular matrix. The extracellular matrix is the term used to describe the proteins and other molecules that exist in the space outside of cells that makes up our body. The extracellular matrix is very important to overall health. In cyst formation, the extracellular matrix provides signals that enable cysts to grow in ADPKD. Non-animal models do not form an extracellular matrix that is present in animal models and so animals are required for this specific project. Non-animal alternatives do not have functioning kidneys that filter toxins out of the blood, and so do not allow for any changes in kidney function to be assessed. This is of paramount importance as ultimately, the maintenance of kidney function is the key outcome of any potential future therapy that involves inhibition of the integrin a1b receptor signalling.

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

Pre-independent feeding zebrafish models of ADPKD

Spheroid cultures

Kidney organoids

Why were they not suitable?

Pre-independent feeding zebrafish models of ADPKD - No model of ADPKD is available in pre- independent feeding zebrafish that recapitulates the human disease. Zebrafish will continue to be used to understand the earliest stages of the disease and how molecular changes, caused by a loss of integrin a1b1, may benefit the disease progression. As such, the use of mice in this project licence application is a partial replacement for the work being performed in pre-independent stage zebrafish embryos.
Spheroid cultures - Spheroid cultures contain cells from a specific region of the kidney called the proximal tubule. However, they do not have flow and do not contain other structures important to the formation of cysts in a fully-formed kidney. As such, spheroid cultures cannot be used for functional analyses and are not comparable to the human kidney.

Kidney organoids - Kidney organoids are a highly valuable tool that have become an important part of the toolkit for many kidney researchers. However, they are restricted by the

fact that they do not filter blood because no bloodflow is present inside them. This means that no physiological functional analysis (such as observing if the kidney is working better) can be performed in kidney organoids. If the integrin a1b1 receptor is discovered to be a viable therapeutic target, kidney organoids might be best used to test specific therapeutic options.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 get statistically significant results we require to perform a number of crosses as we will need to first cross the integrin a1 knockout mice to the ADPKD mice, and then cross these mice in order to begin getting the genotypes we require. Only 25% of the offspring will have the features of ADPKD.

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

As this project is based around breeding and maintenance, we have established a breeding plan that will yield the required numbers of mice with the smallest number of crossings.

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

Efficient breeding will be used to reduce the numbers of mice used in the experiments.

Refinement

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

In this project I will breed two mouse models. These are a mouse depleted of integrin a1 and a mouse ADPKD model. These mice will be crossed together to generate the mouse models required to test the involvement of integrin a1b1 in ADPKD. I will use efficient breeding and maintenance methods to yield these mouse models. Most mice will be humanely killed at 4-weeks of age as this is a time-point when cysts are present in the kidney of ADPKD mice but is before the onset of harmful adverse effects. Some mice will also be grown up to 6 months of age to determine the longevity of the predicted protection

that integrin alpha 1 depletion offers to ADPKD mice. The ADPKD mice I will use can live up to a year, but develop end-stage kidney disease from 9-12 months of age, therefore humane killing ADPKD mice at 6 months will minimise the pain, suffering, distress, and harm associated with this genotype.

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

No models of ADPKD exist in other, less sentient animal models (such as zebrafish).

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

Post-genotyping, all ADPKD mice will be monitored closely for adverse effects such as end-stage kidney disease, abdominal discomfort, kidney infection, dramatic loss of weight, and loss of appetite.

I have refined my experimental design to minimise the welfare cost to the animals. Most animals will be humanely killed at 4-weeks of age, and only a small proportion of ADPKD mice will be grown past this stage, these will be humanely killed at 6-months of age or earlier if signs of adverse effects are observed. This is prior to the onset of end-stage kidney disease and other serious extra-renal abnormalities. Furthermore, the 6-months of age protection experiment will *only* be performed if ADPK mice that have no integrin a1b1 receptor show reduced cyst size and improved kidney function at the 4- weeks life stage. This will minimise the welfare cost to the animals by not performing an experiment needlessly.

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

The NC3Rs website (https://nc3rs.org.uk/3rs-resources) will provide invaluable information regarding best practice guidance, in particular with breeding and maintenance plans. The PREPARE guidelines will also be adhered to (https://norecopa.no/prepare). Their major theme is to ensure proper planning of experiments so as to reduce waste, promote alternatives, and increase reproducibility in research involving animals.

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

Consultation with the staff in the animal facility and with the NC3Rs regional officer will ensure advances in 3Rs are captured. All lab members will be required to use the online resources available at the NC3Rs website before beginning experimentation. In particular, the resources on breeding and colony management will be utilised (https://nc3rs.org.uk/breeding-and-colony-management).
Attendance at 3Rs workshops organised by the animal facility will be expected.

6. Strategies for the restoration of sight in retinal dystrophies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

retina, stem cells, photoreceptors, photoreceptor replacement

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Photoreceptors are the retinal cells in our eyes that transform light into electrical activity. Loss of these cells can result in severe vision impairment and blindness. New photoreceptors can be generated in the laboratory from stem cells. The main aim of this work is to test if the transplantation of new photoreceptors can bring vision back.

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

Why is it important to undertake this work?

Recent estimates indicate that the global number of people with sight loss is 285 million, of whom 39 million are blind. Currently, there are no effective therapies to treat photoreceptor loss; therefore, there is a requirement for new therapies to replace the lost photoreceptors as proposed herein. The results from this work will allow us to test the effectiveness of photoreceptor replacement on the vision. The optimised methods from this work can then

be tested in higher models (e.g., non- human primates) and further translated into clinical trials for patients with photoreceptor cell loss and dysfunction.

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

We would like to see at least two high impact factor publications and at least 6-10 presentations/posters in national and international meetings during this project. We intend to optimise and define the best photoreceptor transplant strategy, which can then be translated in non-human primates and finally to human patients. With this in mind, we aim to be in a strong position to apply for funding to pursue early phase trials in human patients.

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

Blindness has a significant impact on the quality of a person's life often resulting in depression, social isolation and premature death. This poses a major burden to society due to lost productivity and earnings as well as the costs of treatment, rehabilitation and education of the visually impaired and provision of visual aids. Recent estimates indicate that the overall number of people with sight loss is 285 million globally, of whom 39 million are blind. Diseases affecting the retina account for approximately 26% of blindness. The final impact in retinal diseases is the loss of photoreceptors, a specialised type of photosensitive neurons that are capable of light processing. To date there are no treatments to restore lost photoreceptors and visual function, hence there is a pressing need for research into the replacement and/or reactivation of dysfunctional photoreceptors as proposed herein. Successful completion of this project will pave the way for producing and testing photoreceptor transplants in non-human primates and ultimately in human clinical trials. In view of this, the proposed research will benefit first and foremost the patients with retinal disease, their families and carers and society as a whole.

The proposed research will benefit researchers in the fields of retinal sciences in health and disease and clinical applications of vision restoration. Researchers will have an increased understanding of how newly transplanted photoreceptors restore vision in animal models of retinal disease.

Members of the research team will benefit from attending national and international conferences to disseminate results and to maintain existing collaborations and foster new ones. This work will also contribute to the training of several early-career researchers including state-of-the-art molecular, stem cell, and neuroscience and bioinformatics techniques. The proposed work will comprise the excellent further experience of cutting-edge scientific research for the researchers funded by this proposal, providing further development of their careers in academic biomedical research.

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

We plan to disseminate our research findings by presentations at national & international meetings (ARVO Ophthalmic Research conference), Society for Neuroscience (SfN), European Retina Meeting (ERM) and FASEB Retinal Neurobiology and Visual Function meeting. Publication in relevant high impact journals will disseminate our data to academic audiences. We consistently publish original research papers, reviews, and commentaries in relevant journals (Nature Medicine, Stem Cells, Neuron, Nature Communication, Cell Reports, and Biomaterials). We organize national and international networks and societies and maintain regular engagement with patient charities (Macular Disease Society, Fight for Sight, Retina UK, Yorkshire Eye Research, British RP Society and Northern Alliance)

during patient-focused research days. Our Institute and research themes run external and internal seminar series, to which we invite collaborators and experts to present their work and to develop collaborations. Through these meetings and informal contacts, we will maintain existing and foster new collaborations with academic and clinical researchers. We will present at meetings that mark Rare Disease Day, Retina UK information days and we plan meetings to continue discussion of inherited conditions and medical research organised by the Biosciences Institute Public Engagement Committee several times per year. We will contribute to public engagement activities organised by International Centre for Life at Newcastle, British Science Festivals and other university Research Days as well as the charities working in this area (Retina UK, Fight for Sight etc).

Species and numbers of animals expected to be used

- Mice: 613

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 mammals is essential, as the retina of most other species either differs substantially from the human retina from a structural/functional point of view or is too small to allow reliable subretinal cell injections (e.g. zebrafish). Easy breeding and fast development (especially compared to primates) makes mice a valuable tool for performing multiple experiments in which various parameters can be measured with sufficient statistical power within a relatively short time span. Results can then be translated to higher models (for example non-human primates) where animal numbers used for research are lower due to slower development and longer lifespan as well as cost and various ethical considerations. Mouse models of retinal degeneration are widely used to study retinal pathologies and the models to be used in this proposed project have been well characterised by many scientific groups including ours. These studies have shown that these models mimic very well the photoreceptor loss incurred in retinal disease in humans, justifying why they provide the ideal model to perform transplantation-based studies of vision restoration.

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

The animals will be purchased from commercial suppliers or obtained from collaborating labs. Following a period of settlement in our animal facility, photoreceptors will be transplanted in their retinas under general anaesthesia. The animals will be immunosuppressed to stop human cell rejection. Approximately 50% of transplanted mice will be used to investigate how well the new photoreceptors have settled into the mouse retinas. The other 50% will be implanted with a head fixation device and up to two recording chambers. A tracer construct will be injected in their brain visual areas to enable the assessment of light-driven brain activity at the later stages. Behavioural tests (e.g., light avoidance, optomotor reflex and visual cliff), electrophysiological recordings and/or 2-photon microscopy will be carried out on transplanted animals and controls. The animals will be sacrificed at the end of these recording sessions to enable tissue collection for further assessment of cell engraftment in retina and visual activity in brain. Some of the

animals will be dark adapted before sacrifice so their retinas can be used for ex vivo electrophysiological recordings using high density microelectrode arrays.

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

From our previous experience we anticipate that adverse effects due to surgical complications (< 1%: duration 3 days), anaesthesia (1%, duration 3-4 hours), fluid control (< 1%: duration 5 days), injection of genetic construct (1-5% 7 days), implantation of head fixation, indwelling electrodes or recording chambers (1-5%: duration 7 days), stress during behavioural training (~10%: duration 2 days) or recording chamber infection (~10%: duration 7 days) and loss of implant following implantation (5-10% depending on how long the implant has been implanted) may occur. In our recent projects performing subretinal cell injections we have not observed tumour development, abnormal behaviour, weight loss of any other signs of animal distress due to subretinal cell injections. Adverse effects due to surgical complications can be bleeding or infection/inflammation of the wound margin following surgery with associated tenderness and changes in behaviour and body scores. Changes in behaviour and body scores will be monitored as described in our score sheets included in each Protocol. Complications due to anaesthesia can be reduced body temperature and/or slow recovery from anaesthesia.

Complications from implantation of head fixation, indwelling electrodes or recording chambers can be infection/inflammation following surgery with associated tenderness and changes in behaviour and body scores (monitoring see above). Injection of genetic constructs could result in intracerebral bleeds, which could result in changes in behaviour and body scores (monitoring see above). Stress during behavioural training can result in agitation and post testing behavioural changes and body score changes (monitoring see above). Recording chamber infection can occur after implantation and can result in behaviour and body score changes (monitoring see above). Loss of implant would result in exposed bone with possible infection but would always result in termination of the animal. All procedures are of mild or moderate severity.

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

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

The expected severity for both subretinal injections and in vivo electrophysiological cell characterisation is moderate. 95%, 85% and 100% of the animals will undergo this severity in Protocols 1, 2 and 3 respectively.

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?

Both the photoreceptor engraftment and light processing through the eye to retina, optic nerve and finally to visual cortex cannot be performed in vitro, thus in vivo investigations are required to answer these questions. Recording from isolated retinas will allow us to assess restoration of light responses in retinal ganglion cells, the output cells of the retina. It will give us important information on the nature of retinal receptive fields, the range of light sensitivities, and perhaps even colour perception. These experiments cannot be performed on patients, but their outcome will provide extremely valuable new knowledge, paving the way for future clinical trials.

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

We have considered application of retinal organoids as a non-animal alternative.

Why were they not suitable?

Non-animal alternatives are not possible because we work with neural networks involved in visual processing (both at retinal and cortical levels), which can only be investigated 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 was calculated based on experimental design in our funding application to MRC and power stats performed in collaboration with chartered statistician. We have also considered possible losses due to anaesthesia (1%), corneal oedema (7%), surgical complications (1%), subretinal cell injection (20%), fluid control (< 1%), injection of genetic construct (1-5%), implantation of head fixation, indwelling electrodes or recording chambers (1-5%), stress during behavioural training (~10%) or recording chamber infection (~10%).The animals that cannot be used for surgery (for example due to corneal oedema) will be used as controls for immunofluorescence studies.

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

We have performed pilot studies which have shown that 80% of subretinally transplanted mice display engraftment of cone precursors. Of these, 50% of the mice, perform the behavioural tests and 33% of the latter show light sensitivity in at least one eye. These pilot data were used for power calculations performed in close collaboration with chartered statistician, enabling us to assess accurately experimental group size (see below).

Reproducibility and Statistical Design Annex

This proposal will make use of animal models for investigating the engraftment of rod and/or cone photoreceptors and RPE and their ability to restore vision.

To investigate the engraftment of hESCs-derived rod precursors alone and/or in combination with hESC-derived cone precursors and hESC-derived RPE cells at the early and advanced stages of retinal degeneration. The number of mice for each transplanted group is based on our current data with subretinal injections of CRX-GFP+ precursors obtained from day 90 retinal organoids, indicating an 80% success rate in cell engraftment (PMID: 33657251).Given this pilot success rate, to test for a >50% success engraftment rate at a 90% power with a type 1 error rate of α=0.05, a total of 20 animals per group is needed (1 tailed test of proportion, normal approximation).

To assess the impact of hESC-rod transplantation alone and/or in combination with -cones and -RPE cells to restore vision at the early and advanced stages of retinal degeneration. To calculate the number of mice for each group, we rely on our current data, which show that 80% of subretinally transplanted mice show engraftment of CRX-GFP+ precursors. Of these, 50% of the mice, perform the behavioural tests and 33% of the latter show light sensitivity in at least one eye. The number of mice in each class (failed engraftment; engrafted and fail behaviour; engrafted, pass behaviour with no light sensitivity; engrafted, pass and show light sensitivity) are modelled by a multinomial distribution and the marginal distribution of mice that have engrafted cells, perform behavioural tests, and have light sensitivity in at least one eye is binomial with probability 0.132. Our current experience also indicates that 10 mice are needed to obtain sufficient data for recordings from the visual cortex. This procedure requires injection of a AAV5.Syn.GCaMP6f.WPRE.SV40 constructs at multiple sites in V1 and has ~80% efficient (including the follow up transparent window implantation) in our hands, thus requiring 12 injected mice to end up with 10 for visual recording. To obtain an average number of 12 mice that fulfil all the above, a sample of 90 mice is needed. The table of the chance of seeing 6 or less, 7 to 14 and 15 and above is below for one experiment when starting with 90 mice. The combined chance of seeing 7-16 responders is 88%.

Mice	Expected %
6 or less	3.6
7	3.9
8	6.2
9	8.7
10	10.9
11	12.1
12	12.3
13	11.3
14	9.6
15	7.5
16	5.4
17 or more	8.5

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

We will continue to improve the method and efficiency of our transplant protocol. To achieve this, we will keep track of relevant literature in this field reporting on transplant volumes and methods. On the day of transplant, we use a multi team approach where one member performs cell sorting, the second transports the cells to the facility, counts cells and loads them to syringe, thus speeding up the procedure and ensuring cells are not clumping. The third and fourth members of the team will administer anaesthesia and perform subretinal transplants as well as animal monitoring. We will continue to improve this technique and ensure that the cell sorting and prep facility is adjacent to the animal transplant rooms. We will test the effect of hydrogels on cell survival and engraftment with the aim of improving transplant efficiency and reducing animal numbers. We will also share tissue between projects so we can maximise outputs from animal procedures and minimise numbers of animals used. We will work closely with the colony management team to reduce numbers and employ efficient breeding strategies and where possible, we will use both male and female mice to minimise numbers. To maximise animal usage, we will perform several tests on the same animals (e.g., light avoidance, visual cliff, optokinetic reflex, 2-photon imaging), although these will be carefully planned and not performed at the same time.

Refinement

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

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

Most experiments will be performed on genetically modified mouse models of outer retinal degeneration (ORD). The mouse models we plan to use are well recognised for faithfully representing common human ORD disorders and therefore, they will provide important new knowledge about how to rescue or repair retinas with advanced ORD.

The protocols have been designed to keep suffering to a minimum. Where subretinal transplants, tracer injections and recording chamber installation will be carried out, appropriate anaesthetics and analgesics will be administered pre- and post-operatively. Any loss of condition will indicate removal from the procedure and killing by a Schedule 1 method. To define loss of conditions, we will use a score sheet (supplied within each protocol), which considers several features including piloerection, decreased activity, abnormal posture, weight loss, movement impairment, and eye grooming.

All the team members involved in subretinal injections will be trained appropriately by the Vet team and existing team members/collaborators with expertise in this technique. This will ensure competency demonstration which will feed into good animal welfare and consistent animal experience. We will work closely with the Vet team to ensure we are up to date with refinements and improvements in the surgery and anaesthetic protocols.

During the surgery, we will monitor temperature and keep mice close to the oxygen mask until they recover and start to move. Once the surgery is completed, we will monitor animals closely using the animal facility anaesthetic monitoring sheet.

Transplants will be performed using cells that have been differentiated at least for 60 days ensuring that virtually all stem cells have differentiated into retinal cells. To date, we have not observed any tumour formation, but if this occurs, we will work closely with the vet team and report these animals under condition 18.

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

Retinal degeneration occurs over many years in humans; hence, less sentient animals cannot be used as they do not mimic the retina of an adult person. Behavioural tests require in vivo interaction with the environmental stimuli, hence terminally anaesthetised animals are not suitable.

We considered use of alternative animal models, for example zebrafish. Zebrafish models are inappropriate for several reasons: 1) the zebrafish eyes are much smaller compared to mice (radius of retina 1 mm by 13 weeks), hindering subretinal cell injections; 2) although zebrafish models of retinal degeneration exist, all retinal layers are compromised, which prevents studies of photoreceptor integration with host cells that is critical for our studies; 3) zebrafish models of retinal degeneration are very fast and do not mimic the mammalian stagewise differentiation of retinal degeneration observed in mouse models and human patients that underpin the success of donor photoreceptor cell engraftment.

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

We will apply immunosuppression to avoid rejection of transplanted human cells. The immunosuppressant will be administered orally instead of multiple daily injections or inserting mini pumps. We will keep mice in barriered facilities to reduce the likelihood of infections and use anaesthesia during surgical procedures. We are generating in the lab photoreceptor cells that are less "visible" to the host's immune system, thus some animals will be transplanted in the absence of immunosuppression, which has beneficial effects for long term welfare. Best analgesic regime will be optimised in consultation with the vet team and applied before and after the surgical procedure, to reduce pain and suffering. We will use heated pads during anaesthesia and heated cabinets to help with the animal recovery and soft bedding in all cages. Furthermore, to reduce trauma related to surgery, we will use habituation jelly a few days before the surgery. Following the surgery, buprenorphine jelly will be given with the food to avoid additional injections. We have established end points for all adverse effects to reduce animal suffering (please refer to the score sheet supplied with each Protocol).

To minimise infections, we will maintain sterile conditions in our cell preparation methods. Sterility of cultures is checked monthly using established methods. The injection needle is used for one animal and then washed thoroughly in ethanol and PBS before being reused on the next animal.

All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within three-four hours. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health will be assessed by the researcher and the NVS and will be humanely killed by a Schedule 1 method unless a programme of enhanced monitoring and care is instituted until the animal

fully recovers. Any animal not fully recovered from the surgical procedure within 24 hrs (eating, drinking and return to normal behaviour) should be humanely killed. Post- surgical assessment will involve waiting the animal to fully recover after the surgery and daily checks for the first week post-surgery. Afterwards the animals will be regularly assessed by experienced competent animal staff members to monitor for signs of reduced welfare, and if these arise, we will consult the NACWO and NVS for potential treatment and determination of potential endpoints.

For 2-photon imaging animals tunnel handling already established under the AWERB standards will be introduced.

For all models and optional procedures good practice guides will be used to help refine the model as described in the ARRIVE guidelines and Laboratory animal's special article. LASA Working Party guidelines on assessment and control of severity will be used throughout the project to determine if any animal is suffering distress. Should distress occur, immediate actions as described in the individual protocols would be taken to reduce an animal suffering.

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

For all models and optional procedures good practice guides will be used to help refine the model as described in the ARRIVE guidelines and Laboratory animal's special article. LASA Working Party guidelines on assessment and control of severity will be used throughout the project to determine if any animal is suffering distress. Should distress occur, immediate actions as described in the individual protocols would be taken to reduce an animal suffering.

We will follow our statistical design and submit study plans of experiments. We will collaborate closely with NAWCO, NVS and the animal facility staff to ensure best practice. All data will be kept in lab books and university secure servers. Analysed data will be included in our open access publications. We will keep a close eye on emerging publications to avoid duplication of work.

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

We have received funding for our work from NC3Rs and thus obtain regular communications by email about new advances in the 3Rs. We also participate and present in NC3Rs conferences and take part in the regular events (webinars, lunch time seminars etc) organised by our animal facility. We maintain regular contacts with the veterinary team and thus are in a good position to implement advances effectively during the project. We will follow closely work in this field through publication readings, meeting with collaborators and conference participations to ensure we are up to date with refined methods on subretinal cell injections and animal welfare.

7. Aquatic toxicity assessment of chemicals with no or low water solubility

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
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

dietary exposure, acute and chronic toxicity, maternal tranfer, pre-regulatory, chemical hazard assessment

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of the project is to address two key challenges in environmental hazard assessment

The development of a fish test for assessing the aquatic toxicity of chemicals with very low solubility in water (via application of dietary exposures in a series of international OECD test guidelines relevant to regulatory fish toxicity assessment)

A comprehensive toxicity assessment of Dechlorane Plus (one of the chemicals that present very low water solubility) in two generations of 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?

The current regulatory tests for environmental chemical hazard assessment in fish are based on waterborne exposures (e.g. TG203, TG210, TG229, TG230, TG234, TG236). However, many potentially toxic chemicals are highly hydrophobic/lipophilic and therefore present a practical challenge for conducting aquatic animal exposures. In such cases, high volumes of solvents need to be used to achieve solubility or the test fails to meet the acceptance criteria relevant to maintenance of chemical dosing. Solvents present a challenge for discriminating toxicity between the substance in question and the solvent used and in addition, compromise animal welfare. Test failure is resulting is animal wastage. It is therefore important that we address two of the 3Rs (namely reduction and refinement) whilst allowing a sound assessment of chemical toxicity.

Global (including UK) regulatory authorities require chemical toxicity data from vertebrate test systems, including fish, to identify, mitigate and manage any adverse environmental effects arising from their use. The current regulatory tests for environmental chemical hazard assessment in fish are based on waterborne exposures (e.g. TG203, TG210, TG229, TG230, TG234, TG236). However, many potentially toxic chemicals are highly hydrophobic/lipophilic and present a practical challenge for conducting aquatic animal exposures. In such cases, high volumes of solvents need to be used to achieve solubility or the test fails to meet the acceptance criteria for maintaining chemical dose.
Solvents present a challenge for discriminating toxicity of the test substance from the solvent used and may additionally compromise animal welfare. Test failure is resulting in animal wastage. It is therefore important that we address the 3Rs (in this case Reduction and Refinement) whilst allowing a sound assessment of chemical toxicity.

To date, the practical difficulties of conducting aquatic risk assessment for chemicals with no or low solubility in water are acknowledged in only one regulatory test guideline, the fish bioaccumulation test (OECD TG305). Here, for chemicals with a water-octanol coefficient (Kow) >6, a dietary route of exposure is recommended in lieu of aqueous; The Kow is an excellent proxy for hydrophobicity (or lipophilicity). TG305 however, is designed to provide information on chemical bioaccumulation potential alone and does not allow toxicity assessment. In all other acute or chronic regulatory toxicity tests, the dietary exposure option is not present. This creates an issue for regulators considering information from the public domain which often suggests toxicity levels are well above the solubility limit of the chemical.

We intend to develop and subsequently validate a new test method for these substances, which combines the principles of dietary exposure as described in TG305 but also allows toxicity assessment as required by relevant regulatory tests. The model is based on the likely transfer of lipophilic chemicals to the developing embryos via the fish gametes (sperm and eggs). In this way, exposure of the second generation during critical developmental windows, can be achieved via natural transfer rather than the problematic, high solvent-based, aqueous route. This approach would allow toxicity assessment at early life stages, which are critical for environmental risk assessment, as well as chronic toxicity to juvenile and sub-adult stages. For the purposes of test development, it is critical that comparative data between dietary and waterborne exposures are generated. This is because all environmental quality standards are currently based on water (rather than biota) concentrations.
The project aims to combine the principles of existing regulatory guidelines to address two key challenges in environmental hazard assessment: 1) the application of an environmentally relevant route of exposure for highly hydrophobic/lipophilic chemicals (via

dietary exposure of pre-adult fish) and 2) the comprehensive assessment of toxicity of Dechlorane PlusTM (DP) in two generations of fish.

DP is very strongly hydrophobic (Kow above 6) and thus an extremely poorly water-soluble compound. Screening models indicate that DP has a long environmental half-life in water or soil and therefore meets the Persistence criterion of the Stockholm Convention and REACH legislation. As lipophilic compound with a molecular weight below 700, it furthermore has a potential to accumulate in the food web and magnify through trophic levels, thereby are also meeting the Bioaccumulation criterion of the Stockholm Convention.

The third and final criterion for listing a substance as a Persistent Organic Pollutant (POP) under the Stockholm Convention is Toxicity. There is a lack of acute, chronic and sublethal toxicity results for dechloranes, particularly for aquatic organisms. Data on DP toxicity are also scarce; the few available data suggest a low acute toxicity for wildlife or humans. In 2019, Norway submitted a proposal to the Stockholm Convention to list DP as a POP, however, given the lack of evidence on DP toxicity, further evidence is required on which to base a decision.

This project application therefore addresses a long term need to assess aquatic toxicity of chemicals with low solubility on water (aiming at creating an OECD international test guideline following future validation) and a short term need to obtain evidence of chronic toxicity of DP.

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

The main output of this project will be the development of a comprehensive method to assess the aquatic (fish) toxicity of chemicals that are practically insoluble in water (yet require regulatory aquatic risk assessment), thereby protecting animal health and improving environmental quality. More specifically and additionally the project will result in:

- A comprehensive assessment of chronic toxicity of Dechlorane Plus (DP), a chemical that is practically insoluble in water but currently under consideration for classification as a POP (Stockholm Convention).

- A UK-led request to the OECD seeking to update a number of fish toxicity regulatory tests for chemicals of no or low water solubility. The dietary route of exposure is both environmentally relevant and will allow sound assessment of all chemicals of no or low solubility, improving test animal welfare (via reduction of solvents) and reducing test animals used (by avoiding failures to meet dosing criteria).
- A thorough investigation of biomarkers in fish embryos (unregulated life stage) that could be used, following the principles of Adverse Outcome Pathways, as early markers of chronic apical endpoints as required for regulatory decisions.

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

The immediate primary beneficiaries will be the global Environment Agencies.

If the results of this project provide a solid basis for updating a number of test guidelines, then additional beneficiaries will include global regulators and industry who are required to submit the studies.

Ultimately the project looks to improve future hazard and risk assessment of toxic

chemicals in the environment, aiming to protect both human and wildlife health.

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

The applicant is a member the OECD's Validation and Management Group for Ecotoxicity (VMG-Eco), thereby working with the relevant international force that oversees regulatory test guideline development. Following review of the projects' data, a decision will be made on recommending and implementing guideline updates which will benefit regulators and laboratories generating toxicology data in the UK and across the world. In addition, the test design and information on practicalities and benefits will be published in the peer review literature.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 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.

Given the properties of chemicals with low water solubility, exemplified by DP, the main route of exposure to fish can only be via the diet; hence we need to use fish that are capable of independent feeding and employ a dietary route of exposure as suggested for chemicals with high Kow in TG305 (the fish bioaccumulation test).

Data on bioaccumulation and toxicity can be produced for the F0 adult fish (sexually mature) and their offspring (F1), up until they reach sexual maturation. It is important to address toxicity in all these life stages as bioaccumulation (and thus toxicity) is likely affected by the dynamic transport of lipophilic chemicals to the developing gametes; hence a lower toxicity is anticipated in sexually mature fish that can shed the chemical via spawning and a higher in pre-adult life stages.

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

The project involves feeding fish with contaminated diet or exposing fish to the test substance via the water, at a series of concentrations to establish a dose response curve. The duration of exposure will largely follow existing regulatory test guidelines. Following exposure of the F0 generation (adults), the duration of the dietary and waterborne exposure of their offspring (F1 generation), will vary depending on toxicity outcomes. Exposure of F1 generation will continue until sexual differentiation can be assessed with animals briefly anaesthetised at 30dph and/or 60dph to collect weight and length information. We do not expect any adversity at this step other than the stress induced by brief general anaesthesia and recovery. The maximum exposure duration of both the F0 and the F1 generations will be 90 days. At any point of either dietary or waterborne exposures if mortalities/moribundities exceed 20% of what is accepted in the controls as prescribed in the test guidelines these treatments will be terminated. All fish sampled will be killed.

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

project?

On the basis of the available literature, we expect the adverse effects to be mild as chemicals with low solubility in water are typically eliciting chronic, not acute, toxicity. In addition, we intend to use primarily environmentally relevant concentrations, rather than pharmacological doses.

However, given the bio-accumulative nature of hydrophobic/lipophilic chemicals, which is the focus of this project application, we may encounter moderate toxicity as the exposures continue. The clinical signs are difficult to anticipate without any pre-existing information on toxicity and will vary depending on the chemical and/or life stage. For example, we anticipate that accumulation may be higher in juvenile fish towards the end of planned exposures as they don't have the capacity to shed the chemical via spawning. We anticipate additional observations at these later stages and according to the clinical signs detected.

Weight loss, lethargy, abnormal behaviour, altered fecundity and tumours are potentially expected. Death is neither indicated as an endpoint nor anticipated, although there may be cases where high bioaccumulation may result in high internal exposures and potentially death. This is expected to happen only in a small number of fish used as most concentrations employed will be within environmentally relevant levels.

To ensure impact and adversity are kept low/moderate, if mortality/moribundity is >20% of controls at any treatment group, the exposure at this level will be terminated.

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

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

The expected severity is anticipated to be mild for over 50% of the fish used and moderate for up to 50% of the fish used.

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 information this project application is seeking is relevant to fish toxicity; therefore, replacement options are limited to methods that are relevant to fish, excluding for example invertebrate models. It is important to note that the currently available data for DP is for fish, and it is this that really provides the potential concern being put forward by Norway. Therefore, to investigate that we need to use the same taxa, and ideally same species (which we are). The need to use animals stems also from the physico- chemical properties of the chemicals in question, high hydrophobicity and lipophilicity, which suggest that a)

the route of exposure is long term, most likely via the diet and b) potential toxicity is chronic. To date, there are no alternative methods to assess chronic toxicity in fish only acute toxicity.

The planned work includes a comprehensive list of investigative endpoints at the embryo stage (TG236), which if validated as predictive of apical endpoints, can remove the need for using fish beyond this stage, ultimately reducing substantially the numbers of fish needed for toxicity assessment. This includes next generation sequencing and full metabolite profiling of embryos, along with behavioural assessment and apical points (hatching, survival, heartbeat, developmental abnormalities). This powerful approach will advance New Approach Methodologies (NAM's), providing evidence replace downstream chronic toxicity regulatory endpoints, with early, predictive makers of exposure and adversity.

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

We considered TG236 (fish embryo toxicity test) and TG249 (Fish Cell Line Acute Toxicity - The RTgill- W1 cell line assay)

A key project aim is to investigate early and predictive markers in the embryos (we will heavily employ the design of TG236 in our investigations) that can ultimately be used for chronic toxicity assessment replacing fish in the future.

Why were they not suitable?

Non-animal alternatives are not suitable for two reasons:

they are designed to assess acute, not chronic toxicity, which this programme intends to achieve.

low water solubility requires unrealistically high solvent levels, which present toxicity issues for both embryos and 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?

This project is looking at test design that is suitable for regulatory purposes. The factors that were considered in deciding how many fish will be used included:

- The range of concentrations required for establishing a dose response curve (typically 5 although we will consider 3 as a proof of principle)

- Satisfying the numbers of fish used in current TGs for chronic toxicity (including endocrine disruption) to allow future regulatory acceptance

- The variability in assimilation of the chemical into food and subsequent vitellogenin to allow maternal transfer to the embryos

- The fecundity of the species used

- The analytical capability to measure internal concentrations of the chemical in fish tissues (including pools of embryos)

- The current inability to predict chronic toxicity and as such the duration of exposure for the second generation (as required by the current regulatory tests)

The need to generate comparative data between dietary and water exposures as means of anchoring toxicity outcomes using internal concentrations and informing risk assessment which is based on water concentrations.

The potential need to employ additional chemical exposures including positive and negative control chemicals or chemicals that can be diagnostic of mode of action (i.e. co-exposure with antioxidants if oxidative stress is evident from biomarkers of exposure).

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

The experimental design is limited by the prescriptive nature of regulatory OECD test guidelines that allow chronic toxicity assessment. It is key that we provide data that are comparable with these tests as their primary use involves the UK regulatory authority whilst the overall aim is to update TGs (via development of a dietary route of exposure) that will be acceptable by all global regulatory authorities. This can only be achieved if the experiments are robust enough within the current regulatory framework.

A significant reduction of animals is achieved by the addition of a small diversion from the regulatory test guidelines. To obtain length and weight data compliant with a full set of regulatory endpoints; rather than adding additional fish numbers to experiments to enable lethal sub sampling, at critical timepoints fish will be lightly anaesthetised measured and then returned to the experiment until the subsequent timepoint or the end of the experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
Maximising information from the exposures including clinical signs and -omics data (transcriptomics via next generation sequencing and metabolomics) that can be used subsequently to generate information for an Adverse Outcome Pathway (AOP).

The use of zebrafish, a highly accepted regulatory species and a combination of regulatory tests to avoid extensive needs for further validation.

Refinement

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

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

We propose to use a fish species that is a recognised model for regulatory tests for chemical hazard assessment. Zebrafish are recommended and validated for almost all regulatory tests and in addition, most of the available data thus far on DP toxicity are on zebrafish.

Zebrafish are easy to handle, are highly fecund, reach sexual maturity withing 4 months and highly amendable to laboratory culture. In addition, they enjoy many existing resources (full genome sequencing and annotation), facilitating the development of adverse outcome pathways.

The methods will be a combination of existing regulatory tests to allow easy interpretation of data and avoid the need for excessive further validation.

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

Because fish are a key phylogenetic group in chemical risk assessment and can also provide information that is relevant to human health, given the highly conserved toxicity pathways amongst vertebrates. This project requires the use of each development stage from embryo to adult and the long term dietary route of exposure negates the use of terminally anesthetised animals.

Besides, fish are often considered less sentient in comparison to "higher vertebrate" species, so the optimum choice within protected vertebrates.

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

The procedures employed (dietary and/or aqueous exposures to chemicals) are mild in nature and so are the anticipated harms. Toxicity is the only harm that is anticipated but can't be currently predicted as we have little information on the mode of action for most chemicals. The following steps are in place to ensure refinement:
Daily observations (a minimum of two but if signs of toxicity are evident this will increase to a minimum of three) of fish appearance, behaviour and clinical signs via a comprehensive list as developed by the applicant's team for TG203 (see OECD TG203, 2019, Annex).

Application of moribundity in lieu of mortality as an endpoint of chronic toxicity. This is defined by the number and severity of clinical signs.

Termination of exposures in treatments where mortality (moribundity) exceeds acceptable levels by 20% at any point during exposures.

Application of dietary exposure as the **primary exposure route** to avoid the use of high solvent levels, as the latter present potential welfare issues.

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

The applicants team aspires to apply fully the 3Rs principles in their scientific use of

animals. Some key literature that is relevant to the programme and is guiding our experimental approaches is listed below:

CCAC (Canadian Council on Animal Care), 1998: 24 p. [Online]. CCAC Guidelines On: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing. http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/PDFs/APPOPEN.pdf

CEFIC, 2020. LRI-ECO51: Integrating the fish embryo test into the weight of evidence to inform acute fish toxicity. http://cefic-lri.org/request-for-proposals/lri-eco51-integrating-the-fish-embryo-test-into-the- weight-of-evidence-to-inform-acute-fish-toxicity/

Dennison, N., Ryder, K., 2009. The challenges of using humane endpoints in fish research. https://norecopa.no/media/6272/abstract-ryder-endpoints.pdf

Drummond, R.A., Russom, C.L., Geiger, D.L., DeFoe, D.L., 1986. Behavioral and morphological changes in fathead minnow (Pimephales promelas) as diagnostic endpoints for screening chemicals according to mode of action. Aquat. Toxicol. Environ. Fate 9, 415–435.

Ellis, T., Katsiadaki, I., 2021. Clarification of end-points and moribundity for severe (fish) experiments. Laboratory Animals, 55 (3). December 2020.

Hawkins, P., Dennison, N., Goodman, G., Hetherington, S., Llywelyn-Jones, S., Ryder, K., Smith, A.J., 2011. Guidance on the severity classification of scientific procedures involving fish: Report of a Working Group appointed by the Norwegian Consensus-Platform for the Replacement, Reduction and Refinement of animal experiments (Norecopa). Lab. Anim. 45, 219–224.

Goodwin, N., Westall, L., Karp, N.A., Hazlehurst, D., Kovacs, C., Keeble, R., Thompson, P., Collins, R., Bussell, J. 2016. Evaluating and Optimizing Fish Health and Welfare During Experimental Procedures. Zebrafish 13(Suppl 1): S-127–S-131, doi: 10.1089/zeb.2015.1165.

Katsiadaki, I., Ellis, t., Andersen, A., Antczak, P., Blaker, E., Burden, N., Fisher, T., Green, C., Labram, B., Pearson, A., Petersen, K., Pickford, D., Ramsden, C., Rønneseth, A., Ryder, K., Sacker, D., Stevens, C., Watanabe, H., Yamamoto, H., Sewell, F., Hawkins, P., Rufli, H., Handy, R.D., Maynard, S.K., Jacobs,
M.N. 2021. Dying for change: A roadmap to refine the fish acute toxicity test after 40 years of applying a lethal endpoint. Ecotoxicology and Environmental Safety, 223:112585.

Kilkenny, C., Browne, W.J., Cuthill. I.C., Emerson, M., Altman, D.G. 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8:e1000412. Home | ARRIVE Guidelines

McCarty, L.S. 2012. Model validation in aquatic toxicity testing: Implications for regulatory practice. Regulatory Toxicology and Pharmacology 63: 353–362.

OECD, 2019. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures, OECD Series on Testing and Assessment, OECD Publishing, Paris. OECD, 2014. Fish Toxicity Testing Framework, OECD Series on Testing and Assessment, No. 177, OECD Publishing, Paris.

OECD 2013. Test Guideline 210: Fish, Early-life Stage Toxicity Test, OECD Guidelines for

the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD, 2013. Test Guideline 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD 2012. Test Guideline 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure, OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris.

OECD 2011. Test Guideline 234: Fish Sexual Development Test (FSDT), OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD, 2010a. Short Guidance on the Threshold Approach for Acute Fish Toxicity. Series on Testing and Assessment No. 126, OECD, Paris.

OECD, 2010b. Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads, OECD Series on Testing and Assessment, No. 123, OECD Publishing, Paris.

OECD, 2002. Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Human Endpoints for Experimental Animals Used in Safety Evaluation, OECD Series on Testing and Assessment, No. 19, OECD Publishing, Paris.

Percie du Sert, N., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Hurst, V., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Wu¨rbeIl, H. 2020. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol 18(7): e3000411. https://doi.org/10.1371/journal.pbio.3000411. Home | ARRIVE Guidelines

Percie du Sert, N., Bamsey, I., Bate, S.T., Berdoy, M., Clark, R.A., Cuthill, I.C., Fry, D., Karp, N.A., Macleod, M., Moon, L., Stanford, S.C., Lings, B. 2017. The Experimental Design Assistant. Nature Methods Epub Sept 29, 2017. doi:10.1038/nmeth.4462

Percie du Sert, N., Bamsey, I., Bate, S.T., Berdoy, M., Clark, R.A., Cuthill, I.C., Fry, D., Karp, N.A., Macleod, M., Moon, L., Stanford, S.C., Lings, B. 2017. The Experimental Design Assistant. PLoS Biology 15:e2003779. doi:10.1371/journal.pbio.2003779

Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE:guidelines for planning animal research and testing. Lab Anim.

2018;52:135-141. PREPARE (norecopa.no)

Rufli, H., 2012. Introduction of moribund category to OECD fish acute test and its effect on suffering and LC50 values. Environ. Toxicol. Chem. 31, 1107–1112.

Rufli, H., Springer, T.A., 2011. Can we reduce the number of fish in the OECD acute test? Environ. Toxicol. Chem. 30 (4): 1006-1011.

Russell, W.M.S. and Burch, R.L. 1959. The Principles of Humane Experimental Technique, Methuen, London. ISBN 0900767782

Russom, C.L., Bradbury, S.P., Broderius, S.J., Hammermeister, D.E., Drummond, R.A.,

1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 16, 948–967.

Varga, ZM. . 2016. Aquaculture, husbandry, and shipping at the Zebrafish International Resource Center. Methods Cell Biol, 135, 509-534. doi: 10.1016/bs.mcb.2016.01.007.

Varga, Z.M., Lawrence, C., Ekker, S.C., Eisen, J.S. 2016. Universal Healthcare for Zebrafish Zebrafish. 13(Suppl 1): S-1–S-4. doi: 10.1089/zeb.2016.1311.

Wolfensohn, S., LLoyd, M., 2003. Handbook of laboratory animal management and welfare. Blackwell.

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

This body of work is improving current test methods and developing the next steps to meet future regulatory needs and minimise animal use. Engagement with the regulator and up to date good practice on animal welfare, chemical toxicity and regulatory needs will be maintained over the project through close links with the NC3Rs and the OECD Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST), a key force that is providing modern tools for the 21st century toxicology needs. In this context a successful outcome would both refine the testing required and reduce the number of fish used by predicting chronic toxicity outcomes via early molecular initiating events.

8. Surgical Models for Supply 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
    - 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

Surgery, Rat, Mice

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim is to supply high quality surgical models in rodents for use within our orginisation's UK sites. In addition, we aim to bring existing surgical models 'in house' to allow for refinement of these models for use on regulatory and discovery studies. Having a central site for this work will mean that we can refine and improve these models efficiently and produce high quality surgically prepared animals for use on our UK sites.

These surgical models will only be supplied to other projects, within our UK orginisation with authority to use them.

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

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

Why is it important to undertake this work?

This work will allow a centralised surgical service to provide many of the research models required to carry out the regulatory and efficacy work undertaken at our UK sites. A lot of the models we will produce are required for studies that regulatory authorities need to prove a drug is safe to take, or a chemical is safe for use by humans.

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

The outputs will be high quality surgical models for use on studies required by regulators, and other studies supporting this aim. This will help our customers progress their drugs into clinical trials, or prove the chemicals they produce are safe to be used by the public. It is possible that any surgical model refinements that come out of this project may be published and shared with the wider scientific community, to enable better surgical techniques/aftercare to be performed and thus better animal welfare.

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

Centralising small animal surgery at one site will enable us to get a better overview of our small animal surgical services in terms of quality, monitor any issues, apply refinements and improvements in the overall quality of the models, which benefits animal welfare. Customers will ultimately benefit from these outputs as the surgical models will enable them to develop their drugs, prove they are safe for clinical trials, and show that chemicals do not have harmful effects to the public, thus enabling them to sell their products.

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

These models will be used in studies that will inform customers who will, in turn, use it to determine their future strategy, or for submission in documents required by regulatory authorities. These models will be used by our customers in studies to support drugs progressing to clinical trials, or to show that a certain chemical is safe for human exposure, for example. We have previously collaborated with customers and shared data we have produced in the form of scientific publications that are in the public domain. We will follow NC3R's guidelines throughout (National Centre for Replacement, Refinement and Reduction of Animals in Research).

Species and numbers of animals expected to be used

- Mice: 1900
- Rats: 8000

Predicted harms

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

Adult animals will be used in this project, as the testing these animals are being prepared for requires adult species. Rodents are considered to be of the lowest neurophysiological

sensitivity that will allow us to achieve the study aims while being considered suitable for the prediction of effects in humans.

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

Typically an animal will be surgically prepared under a general anaesthetic for use in another project. These surgical preparations will differ depending on the specifics of the study the animal will be subsequently used in. These surgeries include removal of the reproductive organs, the implantation of a cannula to allow administration of test materials, the implantation of a device to allow measurement of blood pressure and heart rate, and cannulation of the bile duct.

Before surgery, the animals will be administered pain relief and possible antibiotics prior to being anaesthetised. After the surgery, the animals will also be given pain relief and maybe antibiotics. They will be initially kept in a warm environment, and may be given special food and water to help their recovery. They will be closely monitored until they show they have recovered. Veterinary guidance will be sought when planning the drugs required for pain relief. After the animals have recovered from the surgery, and a vet is happy with their condition, they may be transferred to other UK projects. Some animals may need to be transported by road to do this. This transfer will be undertaken in appropriate vehicles and under any applicable government regulations. Animals will be transported securely, with food and water provided, in a temperature controlled vehicle. The duration of transport will be the minimum required to deliver the animals safely.

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

Surgical procedures in rodents can result in some adverse effects such as bleeding/blood loss, post- operative pain and weight loss. Most animals have recovered their pre surgical bodyweight fully or partly within 72h of surgery ending. Bleeding is minimised with good surgical technique by an experienced surgeon. Post-operative pain is negated by administration of pre and post-surgical pain relief. If transport is required we will make sure that the animals have recovered sufficiently from their surgical procedures, and have enough food water and bedding for the journey. We will also ensure we transport the animals via the most appropriate direct route, resulting in the shortest possible journey time.

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

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

All animals will experience moderate severity. All animals who undergo a surgical procedure are deemed to have undergone moderate severity by the government.

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?

Surgically altered animals have physical transformations (e.g. implants of catheters/devices or removal of tissues) which can currently not be achieved by chemical or other means. The need to use surgically altered animals will be established as part of the justification process before each internal request is accepted. The internal customer will be requested to provide the required information and relevant sections of their Project Licence prior to surgery being started.

We will be providing animals for projects where replacement has already been considered through the ethical review process of the receiving project licence to which the animals will be transferred.

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

There are no non-animal alternatives to these surgical models. Surgical models cannot be modelled in a test tube or other non-animal model.

Why were they not suitable?

Some of these surgical models are required by regulators after non animal alternatives have identified issues with drugs or chemicals which mean they need to be tested in an animal model. Some of these animals will also be required to test drugs to prove they are safe prior to testing in clinical trials. These types of studies require this testing by law.

Reduction

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

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

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed.

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

Surgically altered animals will only be supplied to specific numbers and orders.

As animals surgically altered under this licence are supplied to a specific request, the principles of experimental design will have already been addressed by the receiving AWERB (local ethical review process).

The numbers of animals produced to fulfil the order and to achieve the scientific aims will be kept to a minimum. Success rates of each model will be under periodic review to ensure that only the minimum number of animals will be implanted. The number of animals surgically prepared will aim to ensure that the study can be completed, allowing for patency issues (i.e. blocked catheters) and other issues that might prevent dosing or sampling. By monitoring closely the success rates of each surgical model we can reduce the number of animals implanted while still ensuring study completion, without the need to repeat a whole experiment.

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

High levels of surgical skill, aseptic techniques and pre and post-operative care will minimise any losses during surgery procedures.

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery.

Before we start surgery, we agree with a Veterinary surgeon what pain relief or antibiotics are appropriate, both before and after the surgery. When recovering from surgery, we give the animals extra heat and monitor them closely until they display normal behaviours. We then check them at least twice daily before they go on study.

In addition, care is taken to provide as much environmental enrichment as possible . This might include plastic shelters in their cages, blocks to gnaw on, extra bedding for warmth and if they need it, food supplements after surgery.

Surgical procedures will be carried out in accordance with the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017). All surgeons will be trained and designated competent by another surgeon who is already competent to assess a specific technique. This will be reflected in their training records.

Refinement

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

Which animal models and methods 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 species and animal model used under this project licence is research demand driven and authorised in the receiving project licence. Materials and methods will be chosen which allow the animals to be as mobile as possible, high standards of enrichments will be provided and the animals will be group housed wherever possible.

The choice of surgical technique or method will always be aligned with best surgical practice. As a minimum this will be in line with published Home Office guidelines.

In order to minimise animal suffering we will administer appropriate pain relief before and after surgery, combined with the most suitable balanced anaesthesia and a high standard of pre and post-operative care, including close supervision by the technicians under the guidance of Veterinary surgeons.

As the Project Licence Holder is part of the Veterinary team, peer review of any major changes/refinements to surgical technique or pain relief/anaesthesia protocols will be carried out by Veterinary colleagues and AWERB when applicable. As always the people caring for these animals are in regular discussion. This group discussion ensures there is no issue from perceived conflict of interest with the Project Licence holder being part of the Veterinary team.

Only the most refined surgical model that meets customer requirements will be prepared.

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

Rodents (rats and mice) will be used in all of the studies conducted under this licence. Rodents are considered to be the species of the lowest neurophysiological sensitivity (have a brain and the physiology similar to humans) that will allow us to achieve the study aims and are considered suitable for the predicting what's likely to happen in humans.

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

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery, and largely to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017).

Before we start any procedures, we also ensure that the requested surgical intervention is required and scientifically justified. We will agree with a Vet what pain relief or antibiotics the animals need both before and after the surgery. When recovering from surgery, we will give the animals extra heat/bedding, and monitor them closely until they recover and start behaving normally again. We then check them at least twice daily before they go on study.

In addition, care is taken to provide as much environmental enrichment as possible . This might include plastic shelters in their cages, blocks to gnaw on, extra bedding for warmth and if they need it, food supplements after surgery.
If we have to transport animals to other sites, this will be done following appropriate government guidelines, ensuring the animals are transported in secure, temperature controlled vans. The animals will be provided with food, water and bedding as appropriate for their journey. The duration of transport will be the minimum required to deliver the animals safely.

Animals will be closely monitored for signs of pain and discomfort after surgery using a clinically appropriate method, for example, The Grimace Scale. Technicians observing animals both before and after surgery are familiar with adverse clinical signs that may indicate pain and discomfort. Animals will be group housed after surgery where possible.

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

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.).

Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Procedures. (2014) London: Her Majesty's Stationary Office.

Home Office Minimum Standards for Aseptic Surgery.

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

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

The choice of surgical model is justified in the Surgical Request form. Any advances in knowledge or refinement surgical techniques will be implemented throughout the lifespan of the Licence.

9. Physiology and pathophysiology of cortical network oscillations

Project duration

5 years 0 months

Project purpose

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

Key words

Brain, Oscillations, Interneuron, Plasticity, Epilepsy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand how network oscillations are generated in the brain, how they influence neuronal communication and learning, and what leads to too much synchronisation of neuronal activity in epileptic seizures.

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

Why is it important to undertake this work?

The human brain is the most complex thing in the known universe, and we still do not understand how this mass of cells gives rise to our thoughts, perceptions and memories. Our work focuses on understanding how the brain works at the level of individual neurons, the connections between these cells, and activity patterns generated in small neuronal networks. We perform these experiments in mice, which have similar cell types and

circuits as observed in the human brain. Importantly, this allows us to use powerful and invasive techniques to interrogate neuronal function, which would not be possible in humans. This is important for generating fundamental insights into brain function, which also supports our understanding of the pathology and treatment of brain disorders. We are particularly interested in what goes wrong in the brain to cause seizure activity observed in epilepsy. This disorder remains resistant to drug treatment in approximately 20 % of patients, and studying the mechanisms underlying such seizure activity in mice will provide the opportunity to identify new targets for treatment.

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

New insights into fundamental neurobiology
Presentations and publications
Insights into the mechanisms and treatment of inherited childhood epilepsy

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

In the short-term, new insights into the mechanisms and function of neuronal network oscillations will be shared with the neuroscience community through seminars and conference presentations.

In the short- and medium-term, the results will be shared with the wider neuroscience community through publications in journals.

In the longer-term, the results may benefit patients with drug resistant epilepsy. We will focus particularly on mice with epilepsy mutations, for which the results would most directly benefit patients harbouring the same or similar mutations. However, there is the potential for wider benefit to patients with other types of epilepsy syndromes.

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

Collaboration - our work focuses on cells and circuits, and we will collaborate with other groups to understand the relevance to behaviour, which will maximise the impact of these studies.

Dissemination of new knowledge - we will publish our results in open access journals and/or deposit the final submitted manuscript in research archives, in order to maximise access. When allowed by the journal, submitted manuscripts will be deposited in preprint repositories to speed up dissemination.

Publication of unsuccessful approaches - graduate students will deposit digital copies of their theses on research archives, providing access to successful and unsuccessful experimental data. Postdoctoral researchers who find negative results will publish these results on an Open Research publishing platform, if this would inform the field.

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.

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

In order to understand the mechanisms and function of brain rhythms, we use mice that have similar cell types and circuits as observed in the human brain. Some of these mice are genetically altered to control the expression of proteins - this allows us to identify, manipulate and/or record neuronal activity in specific cell types. We study the mechanisms and function of brain rhythms during postnatal development, as well as in the adult brain.

In order to understand the causes of childhood epilepsy, we use genetically altered mice that have mutations similar to those observed in patients with inherited neurological conditions associated with childhood seizures. Mice expected to show clinical symptoms are killed before the expected onset of seizures. We thus study the changes in late postnatal development that lead to seizure generation, which is relevant to the pathological progression in inherited forms of childhood epilepsy.

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

The recording of neuronal activity will be performed in mice under terminal anaesthesia, or in tissue harvested from mice that have been killed humanely.

Mice without disease mutations - some of these mice will undergo one surgery for the injection of viruses into the brain, and maintained for periods up to several months to enable viral expression. The mice will then be killed.

Mice with disease mutations - mice will be killed before the expected onset of seizures.

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

Mice without disease mutations are not expected to show any inherent clinical signs. Those undergoing brain surgery are expected show lack of grooming, reduced movement, and redness and swelling around the wound in the 2 days following surgery. Minor weight loss (<10 %) may occur in the week following surgery.

Mice with disease mutations may show deficits in co-ordination and balance during late postnatal development, but this will not affect their ability to eat or drink. Mice will be killed before the expected onset of seizures

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

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

Sub-threshold: 15 %

Mild: 70 %

Moderate: 15 %

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

- Killed

Replacement

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

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

The project addresses fundamental neurobiological principles, which can only be resolved in living brain tissue. The invasive recordings and manipulations of neuronal activity cannot be performed in humans. Neuronal cultures can be produced from immortalised cell lines or induced pluripotent stem cells derived from animals or humans, however these cultures develop under artificial conditions that are not able to replicate the intrinsic and extrinsic inputs that occur naturally during development in situ, and thus display differences in circuit anatomy and physiology.

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

Computational modelling can provide a powerful approach to understand the mechanisms and function of brain rhythms. We complement our studies with computer simulations, but experiments in living brain tissue are required to design and validate these in silico models.

Brain slices can be prepared from human tissue resected during surgery. We are now attempting to complement our animal studies with experiments in human brain slices, but there is limited tissue availability, it is obtained under pathological conditions, and it does not afford the powerful genetic toolbox that can be used to understand circuit physiology in mice.

Neuronal cultures are appropriate for studying the effects of genetic mutations on the molecular biology of neurons, but cannot be used to determine the circuit deficits that develop in situ.

Why were they not suitable?

Non-animal alternatives are not suitable alone to understand physiological and pathological network oscillations, but we use them to complement our studies:

Computational modelling helps in interpreting our data, establishing sufficiency of hypothesised mechanisms/functions, and exploring the parameter space to guide further experimentation.

Experiments in human brain slices will enable us to test whether mechanisms of circuit physiology are conserved between mice and humans.

Results from neuronal cultures will be used to generate hypotheses about the effects of disease mutations at the circuit level.

Reduction

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

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

Each part of the brain contains many different types of neuron, which vary in their anatomical structure and gene expression. These properties cannot be visualised reliably in live brain tissue, and we have previously had to use filling and staining techniques to resolve cell identity after the experiment. The use of transgenic mouse lines enables us to target fluorescent tags to genetically-specified populations of neurons, as well as specifically manipulating their activity, which is a more efficient process in dissecting cellular and circuit function.

The majority of experiments will be performed in ex vivo brain slices, which enables several experiments to be performed in tissue from one animal.

The application of multielectrode recordings and/or population imaging techniques increases the amount of data that can be gathered from each animal.

The majority of our experiments involve acute modulation of neuronal activity, for which we have internal controls. For experiments in which there is chronic manipulation of neuronal activity or expression of disease-related mutations, we will conduct our experiments so that they comply with the ARRIVE guidelines on publication.

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

Efficient breeding - Several groups across our institute use the same genetically altered mice, and we share mice for experiments and breeding to reduce the number of animals used. We reduce the number of breeding pairs to the minimum we can, striking a balance between the numbers required to assure continued genetic integrity and reduction.

Refinement

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

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

Mice without disease mutations, including genetically altered animals for expressing fluorescent proteins, ion channels and/or receptors in specific cell types, are not expected to show any inherent clinical signs. Some of these mice will undergo one surgery for the injection of viruses into the brain - this is required to target manipulations to specific regions of the brain, and is not expected to induce chronic pain, suffering, distress or lasting harm.

Mice with disease mutations to model childhood epilepsy will be killed before the expected onset of seizures. Some of these mice may display deficits in balance and co-ordination, but it is necessary to maintain these mice to the latest possible point prior to seizure onset in order to determine the underlying brain circuit deficits, while limiting the possibility of the mice suffering pain and distress during seizures.

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

While cortical architecture and electroencephalogram (EEG) patterns are well conserved across mammalian species, this is not the case for fish, amphibians and reptiles, and the EEG of non-mammalian vertebrates has proved to have limited use for even distinguishing sleep versus wakefulness. Non-regulated animal species, such as flies and worms, lack cortical structures altogether. The mouse represents one of the least sentient species that can be used study mammalian cortical network oscillations, and is the most refined choice due to its genetic tractability.

The patterns of cortical network activity change during development, and we need to study their mechanisms in both postnatal and adult mice.

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

The protocols we use are well established and scrutinised by the veterinary and research communities, but we shall ensure that we further improve these protocols in the light of any new developments in anaesthesia and surgery.

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

We will follow the guidelines provided by the NC3R (https://nc3rs.org.uk) and Lasa (https://www.lasa.co.uk) on best practice, and follow additional advice provided by the NVS, NACWO and/or Named Information Officer.

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

The project license holders in the department meet every term, and these meetings are attended by the regional NC3R manager who provides updates on any advances in the 3Rs. Further information is provided on the NC3R website (www.nc3rs.org.uk).

10. Breeding livestock for traits derived from new technologies

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
  - 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

CT scanning, phenotypes, genotypes, environmental impact, breeding

Animal types	Life stages
Sheep	juvenile, adult, aged

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The project aims to produce new knowledge and tools to measure traits of importance in breeding programmes for farm animals to increase overall productivity and welfare, whilst minimising environmental impact.

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

Why is it important to undertake this work?

In the long term it is expected that the programme of work will deliver mechanisms to breed sheep for higher production efficiency and welfare, whilst minimising environmental impact. In particular, it will provide in the short-medium term:

novel knowledge on the genetic basis of new traits that could be included in livestock breeding programmes

harmonised and robust methods to measure these traits across large numbers of animals

capability to employ genetic or genomic (DNA-based) selection for traits that have previously proven difficult or expensive to measure, including meat and carcass quality, welfare indicators, feed efficiency, methane emissions.

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

Outputs from the project include:

novel knowledge on the genetic basis of new traits that could be included in livestock breeding programmes. This will include estimation of genetic parameters and genetic correlations to enable recommendations and strategies for inclusion of novel traits into breeding programmes in the most appropriate and effective ways. These results will be published in peer-reviewed journals and presented at academic conferences, as well as forming the basis of articles and presentations aimed at industry end-users.

harmonised and robust methods to measure these traits across large numbers of animals. Protocols and standard operating procedures for phenotyping UK sheep for novel traits will be produced to enable future use of these technologies and methods.

capability to employ genetic or genomic (DNA-based) selection for traits that have previously proven difficult or expensive to measure, such as meat and carcass quality, spine characteristics, health and welfare indicators, feed efficiency and methane emissions. Outputs will include databases, parameters and pipelines to produce routine genetic and genomic breeding values for these novel traits in future.

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

In the medium-long term (5-10 years) it is expected that the programme of work will deliver sheep with genetic potential for higher production efficiency and welfare, but reduced environmental impact. This will provide public-good benefits of producing high quality animal protein, to help meet global food security targets, whilst minimising GHG emissions per kg of product, to help meet net-zero targets.

In the short-medium term it will provide:

novel knowledge on the genetic basis of new traits that could be included in livestock breeding programmes. In the short term, this will benefit the research community and the industry and policy bodies that they inform - to help develop policies and strategies to incorporate novel traits into sheep breeding programmes. In the medium term, this will benefit sheep breeders, who can select for new sustainability traits in their stock, making them more valuable in the market. It will also benefit commercial sheep producers who buy the improved breeding stock to produce lambs that better meet market specifications and government targets for reduced methane emissions (potentially linked to future subsidy payments).

harmonised and robust methods to measure these traits in large numbers of animals. In the short term, this will benefit the research community and industry bodies - to help develop

robust and reliable mechanisms to routinely measure novel traits in UK sheep breeding programmes. In the medium term, this will benefit sheep breeders and commercial sheep producers, as detailed above.

capability to employ genetic or genomic (DNA-based) selection for traits that have previously proven difficult or expensive to measure, such as meat and carcass quality, spine characteristics, health and welfare indicators, feed efficiency and methane emissions. In the short term, this will benefit the research community, potentially across a number of disciplines, and industry bodies - to help develop strategies to incorporate novel traits into sheep breeding programmes. In the medium term, this will benefit sheep breeders, allowing more efficient (and cost-effective) selection of breeding stock of improved genetic merit across a number of important economic and environmental traits, with reduced need for extensive phenotyping of animals. In the medium term, this will also benefit commercial sheep producers, who will be able to buy breeding stock of known genetic merit (with genetic / genomic breeding values) for numerous novel sustainability traits, as detailed above.

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

The research organisation works closely with farmers, industry representatives, funding bodies etc., through its research, education and consultancy activities, and delivers knowledge exchange to different UK and international audiences. They have an excellent track record in applied livestock breeding research and dissemination. Results from previous sheep breeding research projects have been successfully applied in the UK and beyond. For example, breeding values for novel product quality traits measured by imaging techniques, developed under a previous version of this licence, are now provided to all terminal sire sheep breeders that record as part of the AHDB Signet Sheepbreeder scheme across the UK, as a direct result of that research. Therefore, breeding values for these traits are available to the industry on tens of thousands of performance recorded sheep per year. Applied research carried out under this licence will have a similar direct route to end-users within the livestock industry, ensuring maximum impact.

Collaboration with other research groups working in similar areas, particularly within relevant international projects, will result in joint peer-reviewed publications within this field, international harmonisation of data collection protocols and increased impact from combining knowledge and experimental results. These collaborations will also reduce duplication of research efforts, with beneficial effects on total research spend and number of animals used across research trials.

Research results (both positive and negative) will be presented at academic conferences and in peer- reviewed journals, as well as in trade journal articles and at knowledge transfer events aimed at end- users.

Species and numbers of animals expected to be used

- Sheep: 6000

Predicted harms

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

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

Since we need to measure these traits in the target species to enable genetic selection, and look for biological and genetic markers in the relevant species, the work needs to be done on animals and the proposed goals cannot be achieved by using any alternatives. Wherever possible we use our extensive databases containing historical data of archived material from previous studies (e.g. images captured for body composition analysis procedures) or use alternatives such as meat joints, but there are limited opportunities for replacement of animals with alternative models for most elements of the work.

It is anticipated that sheep from a variety of breeds and production systems will be used within the studies, to ensure relevance of research results in different populations of UK sheep. The majority of the trials will focus on growing lambs under 1 year old. However, some studies may also measure mature animals, in order to fully understand the repeatability of results across different life stages, which can have different impacts on the sustainability of sheep systems.

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

Central to the achievement of the project objectives are five experimental procedures: advanced, non- invasive imaging of live sheep for genetic or genomic selection, or to understand interactions with management regime; DNA sampling (e.g. nasal or saliva swabbing, blood sampling, or ear punch) for DNA extraction or to look for biomarkers; individual faecal sampling to look for faecal markers; measurement of greenhouse gas emissions from individual sheep; and sampling of rumen fluid from individual sheep to analyse the gut microbiome. Most animals will only undergo one or two of these procedures once in their lifetime, although some may get multiple procedures over time to look for physiological changes or relationships between measurements. In the vast majority of animals, allprocedures will take place within a period of less than 3 months. Otherwise animals will be managed as in commercial sheep flocks.

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

No adverse effects are expected from these procedures and it is anticipated that animals will be returned to their home flock, and will be fit to be slaughtered or sold for breeding, if 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 animal type)?

All procedures are categorised as mild in their severity level and no animals are expected to suffer anything but short-term mild discomfort or behaviour restriction.

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

- Kept alive
- Rehomed

Replacement

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

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

Since we need to measure these traits in the target species to enable genetic selection, and look for biological and genetic markers in the relevant species, the work needs to be done on animals and the proposed goals cannot be achieved by using any alternatives. Wherever possible we use our extensive databases containing historical data of archived material from previous studies (e.g. images captured for body composition analysis procedures) or use alternatives such as meat joints, but as we are now extending studies into other technologies and traits, there are limited opportunities for replacement for most elements of the work.

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

Wherever possible we use our extensive databases containing historical data of archived material from previous studies (e.g. images captured for body composition analysis procedures) or use alternatives such as meat joints.

Why were they not suitable?

We are now extending studies into other novel technologies and traits, and as such there are limited opportunities to use previously-collected data for replacement for most elements of the work.

Reduction

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

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

The maximum number of animals used has been estimated for the projects that have been funded, or that we hope to receive funding for, within the lifetime of this work programme. The exact numbers of animals required will vary with particular experimental designs and will be based on previous experience and the scientific literature.

For each experiment, an experimental protocol is submitted to the organisational Animal Welfare Ethical Review Body, which includes statisticians in its membership. The protocol includes: a statement of the objective(s) and 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. Furthermore, protocols for each experiment including the above information and other matters such as methods of analysis are produced to conform to Institutional Research Quality Assurance requirements.

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

Consultation with statisticians ensures that the minimum number of animals are used to enable robust scientific conclusions to be drawn. Where possible, on both ethical and economic grounds, our research is based on re-analysis of existing data, or data sharing with other institutes. We maintain well-organised, comprehensive computer-based databases for the type of experiments described here. This means that: (i) historical data is readily available for re-analysis, where this is relevant (thus reducing or replacing the need for animals in some cases), and (ii) we have good procedures for checking whether animals which have been subjected to particular experimental protocols have performed in a different way from others (allowing refinement of procedures if necessary).

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

Where required, pilot studies will be used to provide the baseline data to inform on larger trial work, and to refine sampling procedures and techniques for data capture. We will also use the opportunity to share data and samples with collaborators (internally and externally) to achieve maximum value from minimum resource. In particular, close collaboration with partners in international projects who are using the techniques that are new to our research group (Portable Accumulation Chamber (PAC) measurements and rumen sampling) will inform optimal sample sizes and experimental design. Where possible, results or data will be combined across research groups to maximise impact and avoid duplication of research effort.

Refinement

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

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

Highly trained staff and good animal housing, handling and recovery facilities, have enabled us to refine advanced imaging techniques and tissue / blood /faecal sampling procedures, so that suffering is minimised. We have experienced very few problems in applying protocols very similar to those described here, under our previous licences. Sedation of animals minimises stress. Use of mobile imaging equipment reduces the need for excessive animal transport and means that we can take the equipment to farms with appropriate handling systems. The imaging equipment has been replaced in recent years with a higher specification instrument which gathers data more quickly, reducing the data acquisition time, so that the procedure for each animal is faster and more reliable.

We are constantly looking for minimally invasive ways of collecting samples or data. For example, the use of saliva / or nasal swabbing to replace faecal and blood sampling are used where possible.

For the procedures new to our research group, training will be received from experienced personnel in other countries (Ireland, Norway and New Zealand) or research groups (beef unit technicians within the same organisation) and detailed protocols and SOPs will be shared by those groups, to ensure that methods are employed to minimise suffering. The groups in those countries have recorded thousands of sheep through Portable Accumulation Chamber (PAC) equipment and have rumen sampled hundreds of animals, resulting in highly refined protocols.

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

It is not possible to use immature, less sentient or terminally anaesthetised animals. The study aims to develop phenotyping tools and methods of optimising efficiency and minimising environmental impact that are commercially applicable to farming environments.

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

The procedures described here are already refined as a consequence of our work over the last 24 years and are regularly in use by our team. These include measurements taken by advanced imaging technologies and sampling procedures for blood and faeces. Where we are using new methods - i.e.
methane measurements using portable accumulation chambers or rumen sampling - we will implement pilot testing where appropriate and follow detailed training and instruction protocols from experienced research groups in these techniques.

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

Where appropriate, we will use methods that conform to International Committee for Animal Recording (ICAR) guidelines for recording traits to be used for animal breeding programmes.

The research group has well-established, previously-published, methodology and guidelines for collecting and analysing similar images from live animals using these advanced imaging techniques, and measuring numerous phenotypes from these images. Our group is considered one of the world- leaders in this area of research in sheep.

Published studies, guidelines, SOPs and protocols from highly-regarded research groups, that are leaders in the field, will be followed to ensure best practice for procedures that are new to our research group (portable accumulation chambers and rumen sampling).

When publishing our research we will use the ARRIVE guidelines to ensure that we make all the relevant information known to others and help refine further studies.

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

We will continually review our procedures throughout our work and seek less harmful alternatives whenever these are available. We will regularly visit the NC3Rs website to check for any new developments.

11. Development, homeostasis and disease of the peritoneum and the intestinal tract.

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

peritoneum, post-surgical adhesions, enteric nervous system, aganglionosis, therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to improve our understanding of

the development and role of the peritoneum, especially the mesothelium covering the intestine and the peritoneal wall, during homeostasis and in response to external challenges during (adhesion) scar formation;

the cellular and molecular processes involved in Hirschsprung's disease (HSCR), which is a congenital abnormality of the enteric nervous system (ENS).

We seek to test therapeutic approaches in order to prevent scarring in response to injury (peritoneum), and aganglionosis (Hirschsprung's disease).

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

Why is it important to undertake this work?

The peritoneum is generally well-protected from external injuries. However, a range of clinical symptoms can arise including peritonitis, endometriosis, peritoneal scarring due to dialysis, peritoneal disease (metastasis) and post-surgical adhesions. In all these phenotypes, the mesothelium the outermost layer of the peritoneum, is involved and affected. Post-surgical adhesions arise in response to damage to the peritoneal layers of the intestine and can involve the abdominal wall. Adhesions can arise in up 93% of patients after intra-abdominal or pelvic surgery, leading to considerable morbidity since they can be a major cause of infertility in women, or lead to small bowel obstruction, with a 3-13% mortality rate. Post-surgical adhesions can be a frequent cause of chronic abdominal pain. The only effective treatment is adhesiolysis, which involves further surgical intervention, resulting in a huge economic burden ($2.3Billion / year in the USA). While therapies against adhesion formation include physical barrier consisting of membranes, gels or liquids, none have provided success in randomised clinical trials. Therefore, there is an urgent need to improve our understanding of the cellular and molecular mechanisms that lead to adhesion formation in order to develop successful therapeutic strategies, for example by targeting specific molecular pathways.

Hirschsprung's disease (HSCR) is a congenital disease in 1:5000 live births. HSCR is associated with an absence of enteric ganglia in the hindgut, based on the lack of parasympathetic intrinsic ganglion cells in both submucosal and myenteric plexi of the hindgut. It is thought that HSCR arises as a consequence of premature arrest of the migration of vagal neural crest cells into the hindgut. The internal anal sphincter is invariably affected, however, the absence of enteric ganglia can affect varying length of the intestine. The lack of innervation leads to a distention of the colon (megacolon), resulting in no or very slow gastrointestinal passage. Current treatment involves surgical intervention, with a large number of patients suffering postoperative gastrointestinal dysfunction. However, recent research promises the prospect of cell therapies to regenerate the aganglionic hindgut, however, there are large gaps in our understanding of how therapies using cells or molecules could work successfully.

Therefore, the overarching goal of this project is to establish an advanced understanding of biological mechanisms and to develop translational approaches in preclinical mouse models that replicate the clinical symptoms, for the treatment of peritoneal abnormalities including post-surgical adhesions, and of Hirschsprung’s disease. In order to facilitate these advances in knowledge and understanding, we need to include detailed analyses of the cellular and physiological processes giving rise to mesothelial tissues and their maintenance, as well as communication with neighbouring tissues, and to the ENS.

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

This project will provide a number of outputs, including:
A deeper understanding of external stressors like surgery on the peritoneum. This involves a more detailed insight of peritoneal development and homeostasis, and the effects of ageing on homeostasis and response to injury. In includes defining mesothelial cell heterogeneity and differential molecular responses to injury. These outcomes will provide

novel insight into molecular and cellular processes that could be clinically exploited for therapeutics.

An in-depth analysis of the abnormalities of the ENS in the hindgut/colon of mouse models of HSCR. This will provide us with an improved understanding of the abnormal interactions of cells and the modified underlying molecular pathways in this congenital disease. We will be able to use these insights to determine routes to therapeutic interventions.

Multiple research publications in peer reviewed journals. We have several manuscripts that are going to be published within the coming months and we strive to generate more publications from our funded studies, not only for the benefit of the young researchers involved, but also to improve understanding and knowledge in the field.

Scientists with unique training and expertise in various techniques including surgery, colony maintenance of wildtype and genetically altered animals, approaches to testing possible therapies, data analysis. These skills will be invaluable for the research careers of the staff and students involved in this programme of work.

The long-term aim of this programme is to establish a solid foundation of knowledge of molecular and cellular processes that enable the testing of therapeutic interventions with potential for translation to the clinic.

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

Short term beneficiaries:

The research community seeking to develop treatment for HSCR and post-surgical adhesions, developmental and cell biologists, as well as the regenerative medicine research field. Our research findings will be communicated to these communities via workshops and conference presentations, which will lead to exchange of ideas and cross-fertilisation of outcomes, ideas and concepts. The research communities will also benefit from research publications generated from our findings, with the potential to stimulate further research.

The young researchers working on these projects, as it will equip them with unique expertise in mouse models of injury and disease, mouse genetics, surgery and ex vivo methods arising from these.

Medium to long term beneficiaries:

This will include the research team as short-term outcomes will build further expertise of the entire team, leading to continuity and consolidation as well as refinement of techniques.

The research community (research-interested paediatric surgeons and clinicians and basic researchers, including those interested in HSCR, post-surgical adhesion, developmental and cell biology, regenerative medicine) will be beneficiaries as our findings will increase knowledge. In addition, accumulation and consistency in outcomes will consolidate the reliability and reproducibility of our work, leading to an improvement in standards.

Clinicians (paediatric and adult surgeons, clinicians, HSCR, post-surgical adhesions) will benefit from improved understanding of the mechanisms that lead to the disease outcome, including cellular and molecular processes. We expect that our findings will be important

contributors to the development of clinically relevant therapeutics for treatments in HD or post-surgical adhesion patients that can be tested in clinical trials.

HSCR patients suffering from aganglionosis and blocked gastrointestinal passage, as well as patients suffering from post-surgical adhesions with the associated intestinal obstructions.

The public will benefit from increase in knowledge, dissemination, and treatment options for patients.

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

Our outputs are maximised by interactions with colleagues from preclinical and clinical research fields, as well as interactions with patient representatives. We have ongoing international and national collaborations which strengthen our outputs.

We are strongly engaged with local clinicians treating patients with HSCR and post-surgical adhesions and understand their clinical needs. Through these interactions, we can reach a wide audience of specialist preclinical and clinical researchers and practitioners as well as lay people.

We maximise our outputs through attendance at workshops and conferences, and most importantly through publication of our findings in open access journals, including the use of pre-print serves (e.g. bioRxiv). We are keen to publish unsuccessful approaches, and datasets are made available after publication.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

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

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

Our overarching aim is to develop treatments to prevent peritoneal scarring after abdominal surgery, and to prevent HSCR to cause megacolon and colonic constrictions and associated surgery and morbidities that could in the future be used in patients. We will test cells or cell-derived substances as treatment, and use animals in order to find out which are the most successful cells or substances to reduce the scar in the peritoneum/colonic constrictions. We use mice because their peritoneum and colon have very similar tissue structure and functionality to humans, and show similar response to injury or lack of ENS as in patients. Mice are the simplest animal model with this level of similarity to humans. Therefore, mice are the most appropriate animal to achieve our aims. Peritoneal scarring can occur throughout the life course, often years after surgical intervention. Therefore, we will use adult mice, but also aged mice.

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

Animals in this project may be part of studies that

analyse normal developmental or homeostatic processes, in some cases combined with genetic lineage tracing,

induce injury to the peritoneum after surgery combined with genetic lineage tracing, and/or

test and determine the success of administration of cells or cell-derived substances or molecules.

With these studies, we also wish to understand how the successful treatment works, and where the cells that we use as treatment, go in the body.

During these studies, animals will be administered substances, most frequently tamoxifen or other recombination-inducing agents, to which they may respond with mild weight loss within the 7 days after start of dosing.

Some of the mice will become unwell because they lack gene function. We have specific humane endpoints in place which will allow us to determine when to kill the mice in order to prevent them from undergoing unnecessary suffering.

Mice which undergo surgery, will be under general anaesthesia, but will recover uneventfully from this intervention. They will be given peri- and post-surgical treatment of pain relief. This intervention may be associated with a mild version of weight loss, from which the mice will recover.

Some of these mice will undergo in vivo imaging, which may involve the injection of small volumes of contrast substances that can be detected by the imaging instruments. These steps are typically performed under general anaesthesia. These measurements can take place repeatedly over the study period but at the most appropriate times to reduce harm to the mice.

Some of the mice will receive cells or cell-derived substances or molecules as test treatment. We will monitor and measure clinical signs of progression of the injury or disease with and without treatment.
Mice after lineage tracing induction, surgery and/or therapy treatment may be monitored for up to 6 months.

Some of the mice will be aged when treatment of resumes in order to determine the effect of ageing on injury progression and efficacy of therapies. We have specific scoring documents in place in order to determine the humane endpoint for the mice that are aged and undergo steps of the protocols under this licence, considering age-related frailties.

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

The experimental conditions may lead to loss of weight in mice that

have been given tamoxifen or other recombination-inducing agents,

have undergone surgery,

have undergone in vivo imaging steps.

In B) and C), anaesthesia contributes to the weight loss. Weight loss is only temporary, and animals generally recover uneventfully.

Because of the need for clinical signs of peritoneal injury or colon constrictions in order to assess the effect of the treatments, these adverse effects are necessary.

Aged mice may be more frail than healthy adult mice and their treatment regime during the steps of protocols will be adjusted so that they are milder without preventing us from obtaining relevant information on the same question of peritoneal injury and effectiveness of treatments with cells or cell- derived substances.

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

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

Of the six protocols in this licence, four have a severity band of moderate, while two have a severity band of mild.

In moderate Protocol 2, we may breed genetically modified mice which may show signs of moderate health problems, at about 50% of the animals under this protocol. Ageing mice will be generated under

Protocol 3, which can lead to moderate severity in their clinical signs. Overall, we expect that about 70% of the mice under this protocol show signs of moderate severity.

In moderate Protocol 4, we may perform genetic lineage tracing, which may be combined with surgical induction of peritoneal injury, and administration of therapies to explore their efficacy, and in vivo imaging. We expect that overall, up to 80% of mice under this protocol show signs of moderate severity.

In moderate Protocol 5, we may administer therapies to animals with Hirschsprung's disease and combine this with in vivo imaging. We expect that about 25% of the mice under this protocol show signs of moderate severity.

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

- Killed
- Used in other projects

Replacement

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

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

The conditions that contribute to the development of peritoneal scar formation or Hirschsprung's disease, and possible treatments, cannot currently be reproduced in the non-animal setting since due to the in vivo complexity of the diseases, including by intestinal wall cells, immune cells and other physiological factors beyond the interactions of

cells inside the intestine or peritoneum. Mammalian physiology and anatomy are complex and other organs may also contribute to the injury / disease and any beneficial treatment response. Any beneficial treatment response observed in a non-animals condition may have to be validated using animals models before treatments can be trialled in patients.

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

In vitro systems (cell culture of mesothelial explants or primary mesothelial cells, bowel-derived neurospheres) offer some opportunities to replace the use of mice. Cell culture work is being performed to study effects of therapeutic agents on peritoneal mesothelial or ENS progenitor cells, but only deliver limited information due to the isolated setting. These approaches are currently being used, however, they are based on the isolation of cells or tissues from animals. The in vitro system will be complementary rather than full replacements of animal studies.

Why were they not suitable?

In vitro approaches cannot reproduce the entire disease setting and physiological condition of the animal. These are important aspects that contribute to the injury or disease, and treatment response. This includes interactions with other organ systems and cells as well as physiological parameters including intestinal and peritoneal function. Although cell-based studies produce important information in their limited format on the response of isolated cells to specific conditions, a whole range of these conditions are not being assessed because they still lack the intricate interaction between cells within organs, and between organs and systems in the animal.

In vitro or ex vivo experiments cannot completely replicate the animal model since disease or injury processes in the patient take place in a complex environment. This needs to be considered when working towards achieving the aims of our work, which are to determine the efficacy and mechanisms of action of cells or cell-derived substances or molecules within the animal to peritoneal injury and scarring or colonic constriction based on aganglionosis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experience in this area of research over the last 10 years allows me to estimate with confidence the mouse numbers needed for this programme of work. Animal numbers are determined based on currently funded studies and the experience with previous project licences. These are based on effect sizes (including variances) from previous work or published studies, and also considering adverse effects.

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

The studies funded for this programme of work will have undergone peer review which includes providing detailed statistical analysis and power calculations. We have made use of the NC3Rs experimental design assistant but also other online power calculation tools (G star power etc) to design our studies and power our experiments so that we can achieve sound primary outcome measures. We base calculations on group sizes on estimates of effect sizes from our previous work, including preliminary data, and on published studies.

We have performed longitudinal studies including the use of in vivo imaging, which allow for a reduction of animal numbers since it reduces end point analysis at these different time points.

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

We have established efficient breeding of the compound mutant lines. Out of four genotypes that arise, two genotypes can be used for breeders to maintain the line. One of these can also be used for gene ablation studies. One additional genotype can be used for normal lineage tracing experiments and in combination with surgery to induce peritoneal injury; males of this genotype are mostly used for matings to generate embryos. The fourth genotype is also of value for studies involving only the ubiquitous dTomato reporter expression.

Generally, we attempt to make use of all mice by utilising their tissue post-mortem in ex vivo/in vitro cultures that complement the in vivo work. We have stored tissue blocks which will be of use to students being introduced to techniques of histological analysis including immunostaining.

Image data sets are stored digitally and can be re-analysed at later stages.

We perform pilot studies where required when new external compounds or treatments are being introduced to mice, restricting original pilots to small group sizes (~3 animals).

Refinement

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

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

In this programme of work, we use two different mouse models:

mouse models of surgically induced peritoneal injury and scarring which very closely replicate the clinical phenotypes and symptoms of patients having undergone abdominal surgery. These injuries are required to achieve our overarching aim of developing therapies for treatment of peritoneal scarring and fibrosis, using cell or cell-based or molecular treatments. Our injury models are widely accepted as standard for mouse experiments that

replicate symptoms of peritoneal injury in patients.

These models induce only temporary pain due to the surgery, which will be mitigated with analgesics. In order to analyse contributing cells to scar formation and fibrosis, we include genetic lineage tracing strategies. For this purpose, the mice will be administered a drug/compound (i.e. tamoxifen) that induced recombination to activate lineage tracing of specific cells of the peritoneum. The mice may undergo minor weight loss from which they recover.

Overall, we have not experienced any suffering or distress or lasting harm that in animals of the surgical/lineage tracing model.

mouse models that replicate closely the clinical symptoms of congenital Hirschsprung's Disease. The model is caused by loss of gene function and leads to onset of disease shortly after birth. We have established the humane endpoint as day 7 and until then newborn mice are monitored carefully for any signs of suffering and distress.

Some of the mice in experimental treatment groups will receive versions of cells or cell-derived substances or molecules with the aim to reach a reduction of the level of disease, which should correlate with the level of suffering and distress.

In loss of gene function studies mice will generally be carefully monitored before they reach well established humane endpoints in order for the mice to progress to the clinical signs required to reach the research objectives while undergoing the least distress, suffering and pain.

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

In this programme of work, we study effects of cells or cell-based substances or molecules as therapeutic treatment in mouse models of peritoneal injury and scarring, and Hirschsprung's disease, mimicking variances of the disease found in patients. The mouse is the simplest model organism that can replicate these disease scenarios because it has a very similar anatomy and physiology to the human.

Adult or even aged mice are needed to perform these studies because post-surgical adhesion patients can fall within this bracket. It is necessary to have fully functional organ systems available in the animal for the studies to yield relevant information, and early embryonic stages would not be providing the required data. It is not possible to perform the studies in terminal anaesthetised animals as various outcome measures wouldn't be able to be assessed, making this an impractical approach.

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

I have worked with mouse models of kidney injury for over 10 years, and have experience with mouse models of peritoneal injury for over 5 years.

We started the Hirschsprung's mouse model 2 years ago.

We have also established multi-modal imaging in healthy and injured mice under previous licences.

I have >20 years of experience in mouse models of various diseases, and have used

genetic lineage tracing and ablation models. Based on these experiences, I have established an excellent strategy for monitoring and care of mice by assessing and scoring animal well-being in different disease situations, which could present stages of disease progression and injury.

In order to implement the mouse models of injury and disease, and other procedures within this programme of work, mice are closely monitored during the critical phases after injury induction in order to maintain the severity levels and determine when humane end points are reached.

One of the main predictors of animal distress and suffering is animal weight, and so animals are weighed regularly and their overall appearance monitored every few hours on the day of surgery or other induction of injury, and daily afterwards. This may involve close monitoring of well-being during critical phases of injury response by night visits in order to avoid unnecessary suffering of the mice.

We constantly strive to refine procedures to minimise suffering and improve welfare of the animals. This includes regular reflection of steps that could be improved.

For example, the Hirschsprung's mouse model (Ednrb mutant line) was published as more robust than we have experienced in our animal facility. The clinical signs of lack of an enteric nervous system and subsequent distention of the colon in the Ednrb mutant mouse strain, can lead to severe and life- threatening inflammation of the bowel in the young mice, which had been described as occurring from day 21 onwards. Because Ednrb homozygous mice showed these clinical signs much earlier, between days 7 and 10, we refined our strategy and set day 7 as the humane endpoint for these mice.

In another example, we have optimised the experimental design for surgical induction of acute and chronic kidney injury, since we recognised that animal groups undergo larger variability in response unless certain surgical parameters were more tightly controlled. These observations and refinements have been published on the bioRxiv preprint server and are currently in revision at one of the leading renal physiology journals. This refinement subsequently leads to a reduction in animal number as the experimental design calculations will include adjusted effect sizes, since variability in response doesn't need to be addressed in larger group sizes.

In addition, we have previously established the use of the ultrasound imaging instrument to refine the administration of cells into the left ventricle in order to avoid misinjections and unnecessary suffering.

We plan to incorporate expertise by BSU staff and grimace scoring into an overall strategy that allows us to monitor closely the wellbeing of the mice in an unbiased way.

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

We will follow LASA guidelines on dosing and administration of agents, and withdrawal of blood.

For the scoring of ageing mice, we follow the guidance by Wilkinson and colleagues (Wilkinson et al., 2020, Lab Anim. 54(3), 225-238. DOI: 10.1177/0023677219865291), and also the score sheet that has been set up in collaboration with colleagues who have longstanding expertise in research using ageing mice.

We are in regular contact with colleagues on best practice of the surgical adhesion model as we collaborate and aim to ensure reproducibility between both sites.

We follow the ARRIVE guidelines for which an update (2.0) was published this year (Percie du Sert et al., 2020, PLoS Biol 18(7), e3000410. doi: 10.1371/journal.pbio.3000410).

We have sent researchers to other laboratories nationally and internationally to learn and improve on techniques that are relevant for this programme of work, for example with regards best practice in using in vivo imaging instruments.

As part of a EU Marie Curie training network, that is contributing funding to our work, we are in constant exchange with other researchers in the partner countries to discuss and improve general and specific experimental designs and approaches.

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

Our Biomedical Services team is very active in announcing and organising NC3Rs meetings, workshops and conferences. I have attended several local NC3R workshops, and have presented at an international NC3R conference on severe suffering. I encourage members of the team to use the NC3Rs website, communicate information that becomes available, and attend conferences.

The animal unit at our establishment is proactive in displaying important publications, posters and other information leaflets that are important for work in the NC3Rs area. The NC3Rs regional manager for the establishment is actively engaging with the animal researchers, and happy to provide support where required.

In our work, we constantly aim to apply the 3Rs principles; this includes continuous reflection on studies and subsequent optimisation so that refinements can be introduced to limit suffering, and also to reduce animal numbers. We are also keen to explore alternative model systems (in vitro, organoids, gastruloids, ex vivo etc.) in the laboratory.

12. Development of 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

vaccines, infectious diseases, immunology

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This work aims to develop new and improved vaccines and vaccination regimens for prevention and treatment of infectious diseases (eg malaria, Influenza, HBV, SARS-CoV2) that can be rapidly translated to the clinic. We aim to increase our knowledge of the immune response induced by vaccination and required for protection against infection so that we can further improve our vaccine platforms and rapidly respond to new disease outbreaks.

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

Why is it important to undertake this work?

Vaccines are one of the most cost-effective health tools available. It is estimated that over 3 million lives per year are saved through vaccination, with vaccination campaigns leading to the complete eradication and control of some infectious diseases. Despite these successes, infectious diseases continue to cause significant morbidity and mortality world-

wide because there is no licensed vaccines (eg malaria, Nipah, CCHF, MERs), current vaccines require annual changes (eg Influenza) or the disease is caused by a newly identified pathogen (eg SARs COV2). While historically vaccines were developed for prevention of infectious diseases, the ability of vaccines to stimulate the immune response is leading to development of vaccines to prevent non-communicable disease (eg cancer) but also as treatment tools once disease has developed (eg chronic viral infections). By continuing to develop and optimise different vaccine platforms we are able to apply our technology to a variety of diseases and rapidly respond to new disease outbreaks.

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

Development of new and improved vaccines and vaccination regimens against malaria, influenza and emerging pathogens (eg SARS COV1, SARS COV2, Nipah, MERs, CCHF) that can be rapidly translated to the clinic.

Develop vaccination regimens for treatment of chronic infections (eg HBV, HCV, HTLV-1). Increased understanding of the immune response induced by different vaccine platforms. Increased understanding of the immune response required for protection against different infectious organisms.

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

Short-term impact of this work will be an increase in knowledge and understanding of the immune response following vaccination for the scientific community. Insight into how the immune response is induced and type of immune response required for protection against specific infectious disease will also lead to improvements and alternative approaches to vaccine design.

Medium-term impact of this work will include translation of new/optimised vaccines or vaccination regimens to small scale clinical trials, with long term impact (15 years later or more, typically) includes approval and licensing of a vaccine.

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

Scientific knowledge and results will be disseminated through presentations at scientific meetings (local, national and international) and publication of results in peer-reviewed journals. In addition, we actively participate in local 3Rs meeting and local ethical review committees, sharing and gaining insight into protocol refinement methods. We have strong collaborations with vaccine (human and veterinary) researchers in the UK and international collaborations enabling the sharing of results, resources and techniques.

Species and numbers of animals expected to be used

- Mice: 20500

Predicted harms

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

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

We have chosen mice for these studies as they are the lowest sentient animal, with a complete immune system that closely mimics the human immune system. Performing these studies in mice ensures selection of the most immunogenic and protective vaccines/vaccination regimens that can then be progressed to GMP manufacturing and human clinical trials.

We use juvenile and adult mice as they have a fully developed immune system.

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

The majority of mice will be immunised with a vaccine preparation on up to 2 occasions and killed at a defined timepoint post-vaccination for in depth *ex vivo* characterisation of the immune response. For some infectious diseases, there are established models which mimic human disease, therefore we can test the efficacy of our vaccine. In these instances, mice will be vaccinated with a vaccine preparation and infected with the relevant pathogen (eg malaria or Influenza virus) at set-timepoints post vaccination and monitored for development/clearance of disease.

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

Most animals experience only mild adverse effects following vaccination, in line with the experience of humans (eg mild-flu like symptoms for up to 24 hours after vaccination). In studies testing the efficacy of vaccines against respiratory pathogens (eg Influenza and SARs), the predominant indicator of disease is weight loss, which peaks around 5 days after challenge before mice start to recover. When testing the efficacy of malaria vaccines, we monitor mice for parasites present in the blood, with animals displaying mild systemic signs of disease (piloerection), but for most challenge studies we are able to reach the scientific endpoint of the study before showing systemic signs of disease.

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

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

Mild - 60%

Moderate - 40%

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

- Killed

Replacement

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

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

The immune response to vaccination involves multiple, complex systems interacting in a

physiological environment often involving the antibodies, T cells and cells of the innate system and therefore at present cannot be fully replicated in tissue culture. As our work aims to identify vaccines/vaccination regimens for use in humans, vaccines need to be tested in a system which closely mimics the human body. In addition, mice are the most immunologically characterised species of animal, they have proved to be excellent indicators of immunogenicity enabling the clear assessment of novel vaccines and vaccination regimens. Therefore mice have become the standard species used for testing immunogenicity of vaccines, prior to progression into higher order species if required.

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

Where appropriate we use *in vitro* neutralisation assays (typically lenti-viral assays) as a first step efficacy assessment. This can be used as a surrogate of protection for some diseases (Ebola), typically when there is not an appropriate animal model to robustly test efficacy.

We use an *in vitro* sporozoite inhibition assay that is used to show antibody mediated protection against malaria parasites with mouse, NHP and human serum samples, replacing the need for *in vivo* studies as performed by other groups around the world.

We would also like to explore organoid cultures in our development of an *in vitro* T cell killing assays for malaria.

Why were they not suitable?

These *in vitro* models can only replicate or test one component of the immune system and currently the models are not in place (or sufficient) to fully replicate the immune system, although we continue to explore potential 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 is based on:

- the number of projects we currently have running
- the number of projects for which we are planning to apply for funding
- our experience of how many animals are used within each of our past projects

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

We follow the ARRIVE guidelines and use statistical power calculations to inform and adjust our experimental design.

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

Combining studies: When new vaccines require comparison to standard vaccines, we maximise the number of groups in an experiment, but to the limit of having a manageable experiment where scientific integrity will not be compromised.

Sequential sampling: we are able to monitor the immune response in the same animal over time by either sampling small amounts of blood, or through non-invasive imaging studies. This reduces the total number of animals required for a single study.

Collaborations: Challenge experiments are performed through collaborations with groups that have established challenge models (eg Ebola, Lassa, MERs), therefore we don't need to use additional animals to optimise the challenge models.

Sharing tissue: we collect multiple organs from single experiments and share samples between groups/collaborators when possible.

Efficient Breeding: Breeding will be optimised, wherever possible, to produce only the genotype required e.g. Homozygous breeding pairs, and breeding colonies monitored closely to avoid inefficient and non-productive crosses. Samples for genotyping will be taken in timely manner so that the incorrect genotypes are not kept within the colony.

Using excess mice from breeding: maintenance of our mosquito colony requires feeding mosquitoes with the blood of a naive mouse, where possible we use excess mice from breeding (eg animals that can't be used in experiments) instead of purchasing animals for this purpose.

Pilot studies: when establishing new models, we perform small scale pilot studies to provide important information on reactogenicity of the vaccine, or reproducibility of the assays. This data is then used in power calculations to determine optimal groups sizes required in future experiments.

Refinement

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

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

We have chosen mice are they are one of the most immunologically well characterised animal species and therefore the standard species to for immunogenicity testing of vaccines.

We predominately use non-genetically altered strains of mice, in which the immune response is well defined. When testing the vaccine efficacy, we use strains of mice and species of malaria parasites or viral strains in which the disease course is well defined,

therefore the scientific readout can be reached are earlier timepoints in the disease course.

We use genetically altered mice (GAA) to study the role of specific immune cell-types and genes in the immune response. Alternatively, we use mice expressing a human protein that enabling mimicking aspect of human immune response or is required for infection of mice with human pathogens. We will only use established GAAs of known phenotypes to ensure appropriate care and monitoring protocols are in place.

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

Mice are the least sentient animal with a well-defined immune system that closely mimics the human body, enabling us to test the immunogenicity of our vaccines.

There are extensive sets of reagents available for analysing immune responses in mice and they have proved to be excellent indicators of immunogenicity, enabling the clear assessment of novel vaccines and vaccination regimens for improvements. With the numerous different immunological tools available, mice can be used for detailed characterisation which is not possible in higher organisms. Using genetically modified mice with defined gene modifications provides the unique opportunity to study the role of particular genes in the induction of an immune response and required for protection.

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

We will constantly look for more refined alternative approaches. As the primary aim is clinical translation, we will use adjuvants (or bioequivalent) that have been approved for use in humans. We use the most appropriate route of administration for each vaccine formulation, with routes and maximum volumes following published guidelines.

Our extensive experience with these vaccination studies enables the selection of most informative timepoints.

We use short term anaesthesia when the route of injection can be painful, eg intramuscular injection.

Our typical route of vaccine administration is in-line with standard administration of vaccines in humans (eg intramuscular, intradermal, sub-cutaneous) and only use alternative routes of vaccine administration when targeting the response to specific sites.

Where possible we use less virulent strains of the pathogen and/or modify the challenge dose or route of administration to reduce disease severity.

When studying the role of specific cells requires removal of these cells, generation of chimeric mice (mice that have received a bone marrow transplant) is only used if there is no alternative method that can achieve this scientific aim (eg depletion of cells with drugs or antibodies or the use of GAA mice).

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

The Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn and Maggie Lloyd, Blackwell publishing, Third Edition (2003) in addition to following NC3Rs,

LASA and ARRIVE guidelines.

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

Members of our team are active members of local ethical review committees, therefore regularly exposed to improvements and alternative approaches by researchers using similar models. We receive regulars NC3Rs and University 3Rs newsletters, we attend and participate in local 3Rs meetings. We keep up to date with the literature in both the vaccine and infectious disease field, ensuring we are aware of new or improved models that could be implemented in our research.

13. Tissue response following synovial joint injury and potential for therapies to restore joint structure

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Cartilage, Bone, Osteoarthritis, Cellular therapy, Biomaterials

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to study the biological mechanisms that contribute to the repair response after joint surface injury. This data will provide a better understanding of the role that drugs, biological molecules, biomaterials and regenerative cell therapies can play in restoration of joint tissues.

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

Why is it important to undertake this work?

Arthritis is the biggest cause of pain and disability in the UK. The most common form of arthritis, osteoarthritis (OA) is believed to affect an estimated 8 million people in the UK, and worldwide this figure rises to 343 million. Any joint in the body can be affected by OA

but it is more frequently observed in joints that support the most weight, such as hips and knee. A joint is formed in the body where bones meet and to ensure that they can move against each other, their surface is covered with a smooth, slippery but tough tissue, called cartilage. In OA the cartilage begins to thin and its surface becomes rougher meaning that the frictionless movement of the healthy joint is lost. These changes, together with others in the surrounding bone and lining tissue of the joint can cause pain and loss of mobility. OA is associated with age but younger people are also affected, often impacting on their ability to work, exercise and socialise. Whilst total joint replacement surgery is an option for end stage disease there are no widely available treatments that can be used to halt or ease the progression of OA. Microfracture is a procedure where the surgeon creates a controlled joint surface injury at the site of localised cartilage damage to induce a limited repair response. This approach is currently used as a treatment in a restricted number of patients but produces cartilage which does not completely recreate the original tissue. The goal of our research is to understand how this surgical approach can be refined to benefit a much wider group of patients and produce repair tissue that restores joint function and halts further disease progression.

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

This project will provide greater insight into the healing of bone and cartilage within a joint following injury and how the addition of drugs, cells or materials suitable for introduction to living tissue can improve the healing process. These new discoveries will be shared with others at local, national and international scientific meetings and in widely available publications. The data from this work will form the basis of future funding applications and where appropriate will support patent filings to protect intellectual property. In more detail, it is our expectation that our research will produce one new therapy that can be advanced to a point where it can be investigated further in a large animal model. Over the longer term (5-7 years) we aim to develop at least one human clinical trial to determine the safety and success of the therapies developed in this project.

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

In the early stages of this project (years 1-3), researchers who focus on understanding tissue damage in the joint and the potential to improve the healing response will be the major beneficiaries of this work.
These findings could also have broader significance to those researchers studying mechanisms of disease in the joint and also the wider research community investigating tissue repair following injury. In the medium term (years 3-5), the findings from this work will benefit clinical groups and industrial companies interested in the development of new treatment approaches for the restoration of cartilage and bone in the joint. In the longer term (5-7 years), this work will benefit patients with localised cartilage loss that have been recruited to clinical trials. Ultimately (7-10 years) we hope that larger numbers of patients will benefit from the therapies developed from these studies once they have been shown to be safe and effective.

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

The discoveries from this work, which may include unsuccessful findings, will be shared in the first instance with our research networks. These groups meet regularly and include researchers from broad academic disciplines including life sciences, medicine and engineering as well as complementary areas of biological research. Our results will be disseminated more widely through presentation at national and international research conferences, through publication in peer-reviewed journals and where appropriate

submission to "publish, then review" journals such as eLife or bioRxiv, an open access preprint repository.

Species and numbers of animals expected to be used

- Mice: Mice: 3700

Predicted harms

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

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

This project will use adult mice only. Mice are the lowest experimental species to humans with comparable organ systems and folding limbs that support body weight. This means that our studies on joint surface injury and healing will be performed in a tissue and mechanical environment that provides useful information directly relevant to humans. In addition, mice strains with specific genetic alterations could be used in our work to help identify the detailed mechanisms that are responsible for the healing of bone and cartilage in the joint.

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

In a typical experiment, the mouse will undergo a single surgical procedure to create a joint surface injury in the knee of the animal. This surgical procedure will be completed under general anaesthesia so that the animal will remain in a state of sleep/unconsciousness throughout. The mouse will then be kept alive for up to eight weeks before being humanely killed and tissues harvested for further study. In approximately 30% of the animals a modified material that can be safely introduced into the body will be included at the site of injury to assess its impact on repair. In a further 30% of the animals, a drug or therapy will be administered (orally or by injection, either into a vein, into the body cavity or directly into the joint space of the knee) to determine its potential to modify joint repair following injury. Cells as a therapy will be administered on a single occasion into the joint space whilst drugs may be used on multiple occasions before (for example to target the function of a particular cell type) and/or after the surgical procedure (to directly impact on the healing response). Approximately 15% of animals will have imaging (like x-ray analysis) performed at single or multiple time points whilst the injury is healing. This may include specialist imaging such as magnetic resonance imaging (MRI) to identify cells that have been labelled to allow their visualisation. Detailed analysis of the MRI data will detect altered signal from the labelled cells revealing their specific location within the joint structure. In some experiments, the surgical procedure will expose the mouse knee but not include making a joint surface injury. These so-called, sham-operated mice will allow us to understand the impact of the surgery alone on the biology of the knee joint.

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

The procedure to induce a joint surface injury is generally (in 95% of animals) well tolerated. We anticipate that 100% of the animals that undergo this procedure will have pain, lameness and joint swelling but this will rapidly improve over 48 hours after which

the animals will not show any clinical signs. During this initial 48 hour period animals will receive pain relief. In some cases though, animals may continue to show or develop during the experimental period reduced movement, weight loss, reduced food intake or an abnormal coat. If after interventions, such as treatment with antibiotics or high energy soft food, these clinical signs persist then the animals will be humanely killed.

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

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

Mouse: Mild 10%

Mouse: Moderate 90%

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

- Killed
- Used in other projects

Replacement

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

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

Prior to performing experiments in animals we conduct detailed laboratory studies of the human cells that we judge important in the healing of joint surface injuries, identifying their characteristics and response to factors likely found in the wound environment. However, it is not currently possible using laboratory based approaches to reproduce both the mechanical environment and the complex organisation of tissues and cell types that occur through time in response to injury in the joint. Several of the cell types that are implicated in the repair response only have access to the damaged tissues via blood vessels and so we need a functioning heart and circulatory system to investigate these responses in a way that is clinically relevant and meaningful. Our earlier experience with the experimental approach described here has provided us with a good overview of the healing events after osteochondral injury in the mouse. It has also illustrated the robustness and quality of the data from our studies providing insight into the activity of some cells and the molecules that they may respond to or produce during osteochondral repair. However, further detailed questions remain concerning the function of key cell types following synovial joint injury and the efficacy of therapies. It is these questions that drive the breadth of our planned studies.

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

Approximately 80% of our research is conducted in the laboratory to study individual human cell types both alone and in simple model systems of two or three different cell types. This work includes using the latest cell printing and other manufactured cell culture environments. These studies directly contribute to the replacement of animal studies by identifying the cell types, treatments and time points after injury which should be the focus

of our investigations.

Why were they not suitable?

Even the most advanced laboratory cell culture environment cannot reproduce the mechanical forces and complex tissue and cell interactions that occur within a sophisticated anatomical structure such as the joint. For this reason it remains necessary to address the unanswered questions surrounding the healing response in the joint using animal 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 number of animals has been estimated based on our experience of using these and similar experimental approaches over the last five years. For each of the studies that we wish to perform we are able to predict the number of animals that will be needed to give us reproducible and reliable information. We also understand the lengths of time that our studies need to be performed over to give clear answers to our research questions. This enables us to predict how many experiments can be accomplished over the time given to our studies.

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

Our previous studies have provided us with a clear understanding of the magnitude of effect that is biologically meaningful and its variation within the experimental data. This means that in the experiments that we perform, each experimental group will contain the minimum number of animals to provide biologically meaningful data. When a new treatment, method of imaging or adaptation of the surgically created injury is introduced, it is first investigated in pilot experiments that contain fewer animals. This approach allows us to understand the effectiveness of the intervention and quality of the data to decide if the group sizes in these experiments should be recalculated. We will use the NC3Rs Experimental Design Assistant and other online tools to help us in confirming that our experimental approach is reliable and likely to provide reproducible data.

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

We will use a number of complimentary approaches to reduce the number of animals needed in our work:

Pilot experiments will be performed with fewer animals when a new drug, method of analysis or modification of surgical practice is introduced. This will ensure that subsequent larger studies can be designed to be as accurate as possible.

In studies where the effects of a new treatment or sham surgery versus injury are evaluated, the mice will be randomly allocated to control and treatment groups. In addition, where possible, any treatment will be administered to the animals in such a way that the person delivering it is not aware of whether it is the active treatment or inactive control substance. When the assessment of healing is based on microscopy, "blinded" observers will score the specimens and in experiments analysing large datasets bioinformaticians will analyse the data with no prior knowledge of the experimental groups. The data will then be matched back to the original experimental groups at the end of the analysis, so avoiding bias.

Where appropriate we will share excess breeding animals with other researchers and use any animals culled as tissue donors for use by our group or by other researchers.

In studies that use human cells as a therapy, multiple studies using the same human cells will be performed. This approach minimises variation introduced by using different human donors and reduces the number of animals used.

In some studies, non-invasive imaging techniques will be used to follow aspects of the experimental outcomes of our work through time in individual mice. These approaches mean that fewer animals will be required as it removes the need to cull animals at distinct timepoints.

Refinement

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

Which animal models and methods 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 principal experimental approach that will be used in these studies is a surgically created joint surface injury model. This approach has been refined such that it causes the least pain and suffering to the animals. The surgical procedure itself is expected to result in lameness which will improve within 48 hours. The recovery from surgery will be rapid and progressive with no deterioration in lameness expected to occur. This model is not expected to result in persistent clinical signs that fail to respond to treatment (such as pain relief medication or antibiotics). If however, clinical signs do not resolve animals will be humanely killed and no animal will experience lasting harm.

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

Our work focuses on the joint, a complex part of the anatomy which undergoes significant change throughout development and into adulthood. Adult mice are the least sentient animal that allows us to perform these investigations. The response to injury and the repair processes that follow take days and weeks to develop and for this reason it is impossible to perform these studies under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for the animals?

All animals used in our studies will have a minimum period of 7 days acclimatisation to their environment, including social group, prior to undergoing a surgical procedure. In these experiments each mouse receives a single tissue defect in one knee only which minimises initial pain, discomfort and lameness that the animal might experience. Immediately following the procedure the animals will be housed with access to warmth, post-operative bedding and other nesting materials and wet mash food on the floor to minimise the need for movement. Working with the staff at our animal facilities there are thorough procedures for the post-operative monitoring of all experimental animals, including the use of score sheets to record animal health status, discomfort, lameness and weight. The major clinical impact of our surgical procedure is initial lameness which can be clearly identified by observation but the close monitoring of bodyweight will also help ensure that humane endpoints are adhered to. Oral pain relief medication, that is flavoured to make it more pleasant-tasting, will be given to the mice for several days following surgery.

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

Best practice guidelines including those published by the Laboratory Animal Science Association (LASA), e.g. record keeping, performing surgery, education and training, and reporting of experimental results will be followed in our work. Specific guidance on the principles of surgery will be followed from LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery - A report by the LASA Education, Training and Ethics section (E Lilley and M. Berdoy eds.). The health status of all animals will be supported by guidance on body condition scoring e.g. Ullman-Culleré MH, Foltz CJ. Lab Anim Sci. 1999 Jun;49(3):319-23. In our studies we will also follow the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines on experimental conduct, including study design, avoiding bias and statistical analysis of results.

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

I will continue to review relevant publications, guidelines and best-practice information. This will include resources from The National Centre for the 3Rs (NC3Rs) and practical guidance from the Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA).

14. Developmental control and cell to cell interactions of African trypanosomes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

African trypanosomes, Sleeping sickness, Quorum sensing, Nagana, Development

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to understand how trypanosomes communicate with one another in their mammalian host in order to prepare for their transmission to tsetse flies, the disease vector for African trypanosomiasis (a disease of humans and livestock), and how this is lost in some trypanosomes in the field. We further want to understand how the simultaneous presence of more than one strain or species of trypanosome in a host might influence their virulence or capacity for transmission.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 ability of trypanosomes to use environmental signals via cell-to-cell communication (a process called quorum sensing (QS)) to regulate their development in mammalian hosts controls their ability to prepare for uptake by their vector, the tsetse fly, and so is an important component of disease spread.

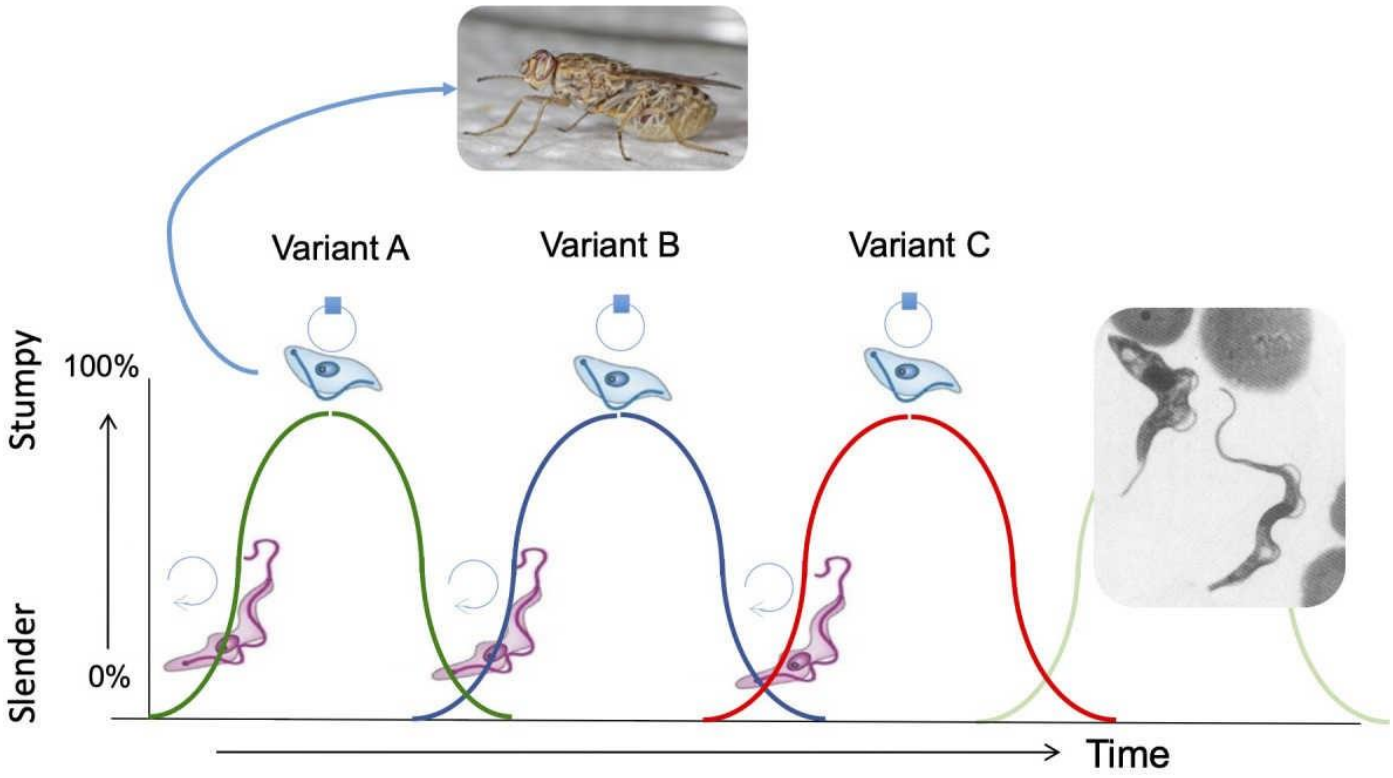


Figure: The life cycle of *Trypanosoma brucei*, showing waves of parasites in the mammal host (caused by waves of antigenic variants in the population), and- within each wave- the development from slender to stumpy forms, ready for transmission. The stumpy forms are taken up by tsetse flies to continue the life cycle.

When the cell-cell communication system is lost naturally in the field, some trypanosome species (Trypanosoma brucei evansi, Trypanosoma brucei equiperdum) have developed alternative routes of transmission using non-tsetse biting flies or venereal transmission, for example. Furthermore, since trypanosomes frequently co-exist with other trypanosome strains and species in disease-endemic regions there is the potential to select for reduced or enhanced transmission and also altered disease virulence generated by interactions between the parasites. By understanding the molecules the parasite uses to monitor and respond to its environment, how these processes are disrupted in some naturally occurring or selected parasite lines or species, and the presence of other trypanosomes, we aim to understand the basic biology of parasite virulence and transmissibility. This also offers the potential to identify routes to block or accelerate development in order to kill the parasites or reduce their transmission and to predict the emergence of more virulent strains. Analysing and perturbing the interactions between trypanosomes also provides the potential to predict the consequences of, for example, drug resistance in the field, or altered host and vector access, for parasite virulence and transmission when in single or mixed infections.

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

We expect to gain new information on the biology of how trypanosomes might perceive environmental information and the presence of other, related, parasites and the

consequences of this as they undergo development as an adaptation for transmission to their tsetse fly vector. Because trypanosomes are evolutionarily divergent organisms, molecular understanding of their mechanisms for perceiving their environment can highlight unusual eukaryotic processes or identify novel mechanisms that differ from those of other eukaryotic organisms including their hosts (humans and animals). This has the potential to provide new knowledge as well as new potential therapeutic approaches, for example through identifying new targets for drugs. The interactions between different infectious agents in a host also has relevance in many disease contexts and our studies will provide new understanding of the consequences of these interactions for disease virulence and spread. The work will generate research publications for the scientific community but will also be disseminated to the public through appropriate channels (e.g., newspapers, radio and television).

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

The discovery of the mechanisms by which trypanosome parasites respond to their environment provides novel routes to controlling the parasite. For example, manipulating the signals that normally drive their cell division arrest could reduce the virulence of the parasite, as could perturbing the activity of molecules that transmit this signal within the cell and between cells. The gene expression profile of some of the molecules linked to arrest as stumpy forms, or proliferation, might also provide molecular markers that permit the prediction of the virulence of the parasite or their likelihood for transmission.

The interactions of different trypanosome species could also be informative in predicting the threat of virulence of parasites that have been selected in coinfected hosts, since they may exhibit particular virulence profiles in the absence of coinfecting competitors. Trypanosomes are amongst the most evolutionarily ancient nucleated cells (eukaryotes). Hence, as well as discovering potential vulnerabilities in their life cycle that can be exploited for therapy, the understanding of their molecular pathways involved in development may provide fundamental knowledge helpful to understand how all cells undergo specialisation, including our own cells.

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

Our laboratory collaborates widely with researchers in the UK, Europe, USA, South America and Africa. These activities generate knowledge sharing and joint publications, personnel exchange, etc. In addition to publication in high impact journals, we have (and will continue) to publish less impactful or negative outcomes in specialist research journals as well as general open access avenues such as BioRxv, Wellcome Open Research, etc. We have also put considerable effort into communicating our research through Schools' outreach, participation and engagement days as well as at a very well attended (at least 37,000 attendees) public museum exhibition.

Species and numbers of animals expected to be used

- Mice: 3500
- Rats: 50

Predicted harms

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

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

We will predominantly use adult mice. Murine infections allow comparison with previous analyses of routine infections in our laboratory; infections in these animals are very reproducible and predictable over the first wave of parasitaemia, which will be the focus of most of our studies. However, some studies will use longer term infections to explore the consequences of long-term interactions between trypanosomes or to compare early and chronic stages of infection. Some experiments will use particular mouse strains where this is necessary for comparison with results from other labs that use that model. Our lab has many years of experience of monitoring trypanosome infections in mice and so are able to identify the likely progression of parasitaemia and associated clinical signs. By accurate projection of the course of infection, monitoring can be increased as necessary during critical phases of infection. The predictability of infections will also aid the selection of appropriate time points to capture parasites at different stages of differentiation. It also minimises the possibility of unanticipated infection pathology ensuring that experiments derive maximum outputs from the minimal number of animals used.

Rats are used when larger numbers of trypanosomes are required- approximately 10 times as many parasites can be harvested from adult rats as from adult mice.

Although in vitro culture of parasites can be used for many experiments, analyses are required where the context of infections in vivo are being explored, for example in the presence of the host immune system or where interactions with host molecules are being studied (for example, degraded host proteins can contribute to the generation of the parasite's quorum sensing signal). Furthermore, the number of trypanosomes that can be isolated for molecular studies from infections is much higher than from culture, such that in vitro methods are not possible for some analyses and become unfeasible where large amounts of parasite material are required for study. Hence, infection-derived material can provide sufficient material to make feasible some molecular purifications that are impossible using culture methods and this is necessary to achieve the scientific objectives of the work.

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

Typically, animals will be inoculated IP or IV with trypanosome parasites, optionally after their immunosuppression with cyclophosphamide. The infections will then be monitored by sampling the tail vein by scab removal after around 3 days until the developmental progression of the parasites occurs in wild type parasites (typically after 6-9 days). Where molecules potentially important in development are identified, mutant parasite lines will be created and their virulence and developmental progression to transmissible stumpy forms will be monitored after ectopic expression, gene deletion or gene silencing, this being carried out with appropriate group sizes and in comparison to wild type parasites.

Most infections will be terminated after 6-9 days when stumpy forms develop, or prior to this if the loss of stumpy formation enhances virulence of the infections. For some experiments longer term infections will be analysed using either single trypanosome species or strains or using a mix of more than one trypanosome species or strain. These infections will be monitored for the relative proportion and developmental status of the component trypanosome species/strains.

The distribution of parasites within the mouse host may also be monitored using red-shifted luminescence imaging under anaesthesia. This involves the inoculation of parasites

IP, and then the monitoring of infections at time points after infection under anaesthesia by the inoculation of a luminescent substrate to allow the compartment or distribution of the parasites to be visualised using recording equipment.

Where pharmacological agents are identified that may alter the development or viability of the parasite, these will be investigated for their effects on parasite viability, development or growth in vivo. This will be preceded by range-finding experiments where appropriate pharmacological doses are determined. In each case, parasite numbers and developmental form will be assessed by isolation of a blood drop from the tail vein and then analysis of parasite numbers or morphology microscopically or using molecular markers (e.g. immunofluorescence microscopy, qRT-PCR).

The transmission capacity of parasites may also be monitored by assessing the ability of tsetse flies to become infected after feeding on anaesthetised infected mice.

In all cases, at the end of experiments, animals will be terminally anaesthetised and, optionally, parasites harvested by cardiac puncture and blood collection. Organs might also be isolated post mortem to determine the distribution of parasites according to the luminescence of tissues infected with parasites expressing red-shifted luciferase.

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

Trypanosome infections can be fatal in rodents, death being preceded by progression through a predictable series of symptoms on a relatively predictable timescale. Our experience of working with these parasites in mice (over 20 years) means that we have become expert at monitoring and predicting the progression of infections, so that experimental outcomes can achieved without the infection leading to severe illness or death, which is a rare occurrence (less than 0.5% of infections). Mice and rats are monitored for their disease progression based on a numerical scoring system and undue suffering prevented by humane killing should the infection progress to a level where death is anticipated within a few hours or might occur if animals were left unmonitored overnight. As a consequence, the overall severity for our experiments is classed as moderate. At the end of experiments animals are euthanized and parasites are harvested.

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

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

Mice- expected severity based on values from 2020 Mild- 32%
Moderate- 68%

Severe- less than 0.5%

Rats- expected severity (no experiments with rats were carried out in 2020, values are estimates based on historical values)

Mild- 50%

Moderate- 50%

Severe- less than 0.5%

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

- Killed

Replacement

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

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

We routinely grow trypanosomes in cell culture. However, their life cycle development does not progress normally in cell culture, such that the production of the transmission stage (stumpy forms) is best achieved in parasites grown in mice. Furthermore, the evaluation of the effects of disrupting particular parasite molecules and processes can only be accurately determined by studying parasites growing in the natural context of a bloodstream infection in a mammalian host where the host physiology and immune response may contribute. For the isolation of large numbers of parasites, the ability to harvest tens of millions of parasites from a single infection makes some experimental approaches feasible that would not be possible using cultured parasites that are in an un-natural growth medium and isolated from the host (and thereby in the absence of an immune response and where molecular signals are not at natural levels due to their non-physiological accumulation or turnover in culture). Furthermore, parasites grown in culture do not generate synchronous populations of stumpy forms that are required to analyse molecular events in the population that reflect molecular events going on in each individual parasite. Coinfections also require to be studied in the context of a functional immune system. Whilst interactions between parasites in culture can be informative, ultimate validation of any effects observed needs to carried out using parasites growing in a mammalian host where the combination of immunity, signal production and turnover can contribute to the infection dynamic. Where parasites occupy particular host body compartments, this must also be assayed in vivo.

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

We have developed non-animal based protocols for growing parasites and generating stumpy forms in vitro using brain heart infusion broth and, more recently, basement membrane extract. These are effective at generating populations of stumpy forms that accurately reflect those generated in vivo. This allows genetic perturbations of parasite lines to be evaluated in pilot experiments and the onward developmental capacity of trypanosomes to tsetse midgut forms to be explored. Small molecules driving or inhibiting differentiation can also be tested in these in vitro assays. However, these cannot fully recapitulate growth in vivo, and so experiments must also be validated in vivo or extended to assay the contribution of the host physiological environment.

Why were they not suitable?

In vitro culture cannot accurately reflect the complexity of in vivo signals, parasite interactions with host molecules or the immune system, or the kinetics of small molecule accumulation or turnover in vivo. In addition, the contribution of physiological compartmentation (e.g. in the adipose tissue or skin) cannot be studied in vitro. For these

reasons assays finally must be carried out in vivo to assess the environment in which parasites establish and differentiate accurately.

Reduction

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

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

The numbers are based on current laboratory usage and reflect the number of animals needed to generate molecular material or maintain parasite blood stocks, or the number of animals required for statistical validity when assessing parasite phenotypes after gene knockout, knockdown or overexpression studies. Likewise, experimental design considerations for the analysis of coinfection experiments dictate the numbers of animals used in experimental groups. In all cases, pilot assays are also used to establish the dose of trypanosomes for infections, their likely progression of growth and differentiation and dosages for particular pharmacological regimens. These small pilot studies help to optimise the experimental studies where larger groups of animals are used for statistical analysis.
Maintenance of parasite blood stocks (which can be cryopreserved) can also use small animal numbers.

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

Trypanosome growth in mice progresses on a relatively predictable course, such that it is straightforward to generate reproducible and statistically valid datasets that satisfy external scientific scrutiny (i.e. where the probability of the observed outcome being incorrect is less than 5%). We are experienced in analysing and predicting parasite virulence in mice and so can minimise distress whilst deriving the necessary scientific information from infections. Genetic manipulations of trypanosomes often involve use of gene silencing or gene overexpression techniques, which are controllable using chemicals supplied in the rodent drinking water (e.g. doxycycline). This provides well controlled analyses because phenotypic comparisons between treated and untreated populations provide a robust experimental outcome using the same parasite material. We have used the assistance of an Experimental study design and statistical support officer to help plan appropriate group sizes for studies where there is variance in the effect size. This support provides advice on the planning and interpretation of experiments to achieve statistically meaningful outputs.

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

Pilot assays are used to establish the dose of trypanosomes for infections, their likely progression of growth and differentiation and dosages for particular pharmacological or experimental regimens. These pilot studies help to optimise the experimental studies where groups of animals are used for statistical analysis.

In vitro assays using basement membrane culture media also optimise our studies by identifying those cell lines that exhibit the appropriate gene perturbation or best regulation using inducible systems such as tetracycline/doxycycline controlled overexpression or gene silencing.

Refinement

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

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

Mice are used to monitor trypanosome infections in laboratories worldwide. This allows comparisons of the infection profile and kinetics of proliferation and differentiation between studies and between laboratories. The symptoms linked to trypanosome infection in mice are also predictable allowing us to track the progression of infections with a scoring system allowing defined humane end-points. A staged approach will be adopted when novel drugs are used. Mice do not exhibit overt signs of distress or discomfort from infection except in the critical phases when infections are closely monitored.

Rats are required occasionally where it is important to isolate very large amounts of parasite material- beyond that achievable either in vitro or in mouse infections.

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

Trypanosomes can only infect mammalian hosts as their disease relevant bloodstream form. Infections must be sustained over a period of days to allow for parasite development, or longer to explore aspects of the parasite's interactions with the host or other parasite strains or species. Adult animals are required to explore the parasites' biology in a manner relevant for livestock or human infections in Africa.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?
We carry out ongoing review of procedures, in consultation with the relevant services at the Institute, to assess where animal welfare can be ensured and enhanced. Examples of procedure adaptations include improved environmental enrichment, avoiding single housing where possible and exploring the potential for the introduction of refined local anaesthesia for the harvesting of small parasite samples from the tail vein etc.

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

Experimental procedures have been developed in house but also adapted or adopted as new approaches are described or detailed in the literature. For example, we have published methodology for parasite development in vitro using Basement membrane extract. Other methodologies follow those of colleagues in other laboratories.

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

Ongoing training and education will be provided through the Institute, with updated information provided via their regular community meetings and information updates. Training guidance will be disseminated through local animal unit personnel and at our own laboratory meetings, held weekly.

15. Neural circuits: neuron-glia-immune interactions 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

Neuroscience, Inflammation, Psychiatric illness, Memory, Dementia

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to study brain function in normal, healthy conditions and how this changes due to disease. We will also study how life-style 'risk factors' such as obesity interact with the **immune** system to increase the severity of conditions such as dementia. Our focus is on different cell types in the brain: **neurons**, which are nerve cells that communicate via electrical signalling, and **glia**, which form the majority of cells in the brain but were previously viewed as 'care-takers' and mostly ignored by researchers. Recent advances show that glia actually play a profound role in sculpting brain structure and function, and may be responsible for mediating many healthy and disease-related processes in the brain. Additionally, we aim to test the idea that disruptions in **neuron-glia-immune** interactions are a common feature that underlie many neurological and psychiatric illness.

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

be short-term benefits within 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 **neuron-glia-immune** interactions is likely to be the key to unravelling the neurobiology of many diseases. The emerging field of **neuroimmunology** has demonstrated that there is a life-long, continuous interaction between brain and immune systems that begins to affect brain function even at the earliest stages of pregnancy. Molecules that the immune system uses to signal throughout the body are also involved in determining how the brain develops, from embryo to adulthood. The aim of our project is therefore to study electrical communication in the brain to determine how different regions interact to support brain functions such as memory and decision-making, and how interactions with the immune system change this.

Immune molecules are produced to excess in conditions such as obesity, smoking and lack of exercise; these conditions appear to greatly increase the risk of late-life disorders such as Alzheimer's disease and other dementias, by creating an excessive amount of immune molecules in the body, causing inflammation. These inflammatory molecules from the blood then enter the brain and are known to activate certain glial cells, called **microglia**, and affect how microglia can clear toxic proteins and repair damage in the brain.

Much neuroscience research is focused on neurons. However, it is important to understand how these neurons are changing in the wider context of the other cells in the brain. By learning how neuronal function is shaped by glial cells and influenced by the immune system, we believe that we will gain important insights into the mechanisms through which an individual's lived experience and lifestyle factors can interact with their genetic background, making them either more or less susceptible to disease. This should uncover novel potential therapeutic targets for prophylactic or disease-modifying therapies.

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

By the end of this project, we believe that we will have learned a great deal of information about how immune system activation at different points in life (pregnancy, adolescence, adulthood) interact with genetic risk factors to manifest as neurological or psychiatric disorders, with mechanisms including changes in the brain's electrical activity, and how the brain's immune cells can shape connections between different neurons throughout life. We also expect to gain important insights into how these systems interact in normal, healthy conditions. Beyond new knowledge, the outputs from these projects will mainly be in the form of scientific publications, and lectures to academics and the public.

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

In the short-term, the benefits from these outputs will be increased understanding of how the immune and brain systems interact in healthy and diseased states. In the longer term, this could provide novel therapeutic targets for the treatment of psychiatric or neurological conditions, from schizophrenia, through depression to Alzheimer's disease and Lewy Body dementia. Mental illness such as depression affects 1 in 6 people at some point in their lifetime, with 1% of the global population being affected by schizophrenia. As humans live longer, dementia is becoming an increasing burden on society: there are 900,000 people in the UK living with dementia (out of 55 million worldwide) at a cost of almost £35bn per year. By 2040, it is estimated that 1.4 million individuals in the UK will be living with

dementia, at a predicted cost of over £94bn per year. If our research can provide new therapeutic targets for treatment of these conditions, the potential impact is significant. By studying how the immune system interacts with the brain, which is implicated in many distinct brain disorders, our research will have relevance to all of these conditions.

Furthermore, our plans to replace immune cells in mice with those derived from patients could provide key information in predicting which patients are more likely to show improved response to medications, using preclinical research as a stepping-stone to precision, individualised medicine.

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

In order to maximise the benefits of our work, I have established collaborations with clinical colleagues working in the field of rheumatology; rheumatology patients are at an increased risk of developing conditions such as depression and dementia, so by working collaboratively in projects that incorporate both human clinical, and our preclinical mechanistic studies, we hope to maximise the potential of our work to play a role in improving human life. We have also established collaborations with neuropathologists, where we have been using insights from human post mortem studies to better improve our approach to developing animal models of dementia, particularly in the field of Lewy Body Dementia. Additionally, we are currently actively exploring opportunities for collaborations with pharmaceutical companies in the field of neuroinflammation. Whenever not commercially-sensitive, we will publish our findings as widely as possible, including depositing our results on preprint servers upon submission to academic journals.

Species and numbers of animals expected to be used

- Mice: 10500
- Rats: 350

Predicted harms

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

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

We will primarily use mice because a number of advanced tools for studying brain function have been developed in genetically-altered mice, and because well-established models of both Alzheimer's disease and schizophrenia have been developed in genetically-altered mice. Our choice of life stages are dictated by experimental need: to study how environmental factors during pregnancy can influence brain function over time, it is necessary to work with pregnant mice. Similarly, dementia is a late-life disease, so it is necessary to study this disorder in older animals. Most of our work will be carried out in young, adult animals. Rats are more intelligent than mice and are larger, so we may carry out a small number of experiments in rats when these attributes make them more likely to be able to successfully complete an experiment.

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

Many of our experiments will require surgery where we can use viruses to insert genes into various parts of the animal brain to allow us to change the way that they function, or to

better study how different parts of the brain are connected. In these cases, animals will undergo surgery lasting around an hour, where we make a very small hole in the skull to insert a small needle to inject the virus into the brain.
We will then seal up the skull and allow the animals to recover. Typically, animals experience some pain after the surgery, but this can be easily controlled with medication, under the guidance of a vet. Animals typically recover within a few days, and are housed normally for several weeks until they are killed under deep anaesthesia so that the brain can then be removed for further study.

In a smaller group of experiments, we will also implant devices into the animals' brain to allow us to record electrical activity while they carry out behaviours such as exploring a maze. During these surgeries (typically lasting 2 hours), animals will be deeply anaesthetised and small devices (typically weighing around 2 grams, which is about 10% of a mouse's body weight) will be implanted into their brains and secured in place using dental cement. At least a week after surgery, animals will undergo behavioural experiments, during which they will be connected to recording apparatus via a wire, which can cause some initial distress. Animals quickly get used to this type of experiment, and become comfortable enough to fall asleep during the experiments. Before each behavioural experiment, animals may receive an injection of a drug designed to increase or decrease activity in a specific part of the brain to allow us to better understand how that part of the brain is needed to carry out the task. At the end of these experiments, animals will be killed under deep anaesthesia to allow us to remove the brain and study anatomical changes. Alternatively, instead of using electrical recordings of brain activity, animals will have a small glass window implanted in their brain to allow us to use microscopes to directly observe activity in the brain. When connected to the microscope, the heads of the animals will be fixed in place, but they will be able to move by running on a treadmill or floating ball, which minimises stress from the procedure.

Some of our experiments require us to examine how lifestyle factors affect the development of brain diseases. In these case, mice will be fed a high-fat diet to mimic lifestyle factors known to increase the risk of brain disorders in humans.

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

Animals that have been genetically modified to model diseases such as dementia or schizophrenia show only subtle changes in behaviour or memory that require special tests to be able to observe. The most common adverse effect for our animals will be pain immediately after undergoing surgery to have viruses injected into the brain to deliver genetic tools and / or devices implanted for recording brain activity. Animals typically require painkillers for 1 to 2 days after surgery, under guidance from our vets, and fully recover within 7 days. Animals with head implants typically experience some distress when first connected to recording equipment, but get used to these after 2 to 3 recording sessions. When not connected to recording equipment, animals typically show no signs of distress or discomfort due to the presence of head implants.
In a smaller number of animals, we will inject them with substances designed to mimic viral or bacterial infections, to study the effect of the immune response on the brain. In these cases, animals will typically feel unwell for a few days after the injection.

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

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

(per animal type)?

Most (around 60%) animals will be genetically-modified animals that are bred for the sole purpose of creating more mice (some of our experiments require two different types of mouse to be crossed to generate those useful for experiments). These mice will experience only mild severity, although there will be no aversive phenotype beyond subtle changes in behaviour in some of the transgenic disease models that require sensitive tests in order to observe these behavioural changes.

The remaining 40% of mice will experience moderate suffering due to the experiments described above, as will 100% of rats. The majority of experiments will be carried out using mice, with rats comprising less than 5% of all animals used. Of these animals The remaining 20% of mice will undergo a viral injection as well as a brain::

- Approximately 75% of mice (and 60% of rats) will experience only a viral injection into the brain and will recover fully within 4 - 7 days. After several weeks or months to allow genes delivered via viruses to be fully expressed, these mice will be made unconscious (using an anaesthetic) and their brains collected for further study without additional suffering.

- Approximately 15% mice (and 40% of rats) will receive a head implant and / or a viral injection.

- Approximately 10% of mice in total will receive a manipulation to activate the immune system (either a high fat diet, genetic expression of immune molecules, or injection with substances to mimic viral or bacterial infection), with or without viral injection and / or head implants.

- more than 80% will experience only a viral injection into the brain, and will recover fully within 4 - 7 days. After several weeks or months to allow genes delivered via viruses to be fully expressed, these mice will be made unconscious (using an anaesthetic) and their brains collected for further study without additional suffering.

- Of the remaining 20% of mice, fewer than half will undergo a viral injection as well as receive a head implant as described above.

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

- Killed
- Used in other projects

Replacement

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

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

Our research aims to discover how different regions of the brain are *functionally* connected in both health and disease, and how different types of brain cell are influenced by the immune system. While we can learn which brain regions are anatomically connected using histological studies in dead tissue, these studies cannot tell us how these anatomical studies influence electrical activity in the brain, nor how this electrical activity can generate

complex behaviours such as learning and memory. Only by studying live animals can we discover how activity from different types of brain or immune cell can influence behaviour. Additionally, our focus is on how long-term increases in immune activity, through lifestyle factors such as poor diet, can change activity in the brain and interact with the progression of diseases such as dementia both at the level of brain circuits and behaviour. As a late-life disease, we can only study these interactions in organisms that live sufficiently long, have an immune system analogous to humans, and have a nervous system sufficiently complex to support experimental studies of decision-making and memory.

We are interested in the complex connections linking different parts of the brain, so need to study an animal that has analogous regions to those in humans. As such, mammalian models are essential for this work as non-protected animals such as fruit flies and nematode worms do not possess sufficiently complex brains. Much of our work will be carried out in rodents: qualitatively, rodents have the same brain regions as humans, but quantitatively, the brain is smaller and simpler. However, the brain regions that subserve memory and decision-making in humans (hippocampus, prefrontal cortex, etc) carry out the same role in rodents and no substantial differences between humans and rodents in terms of connectivity or basic circuit physiology have yet been discovered. Furthermore, numerous genetic tools for studying the function of brain circuitry exist in genetically-altered mice, so using mice will enable us to take advantage of these tools.

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

The only non-animal systems that could potentially be useful in this project are computational modelling or *in vitro* systems such as cell culture or the use of brain organoids (small 'brains-in-a-dish' that have recently been described).

Why were they not suitable?

Computational (*in silico*) approaches are not suitable because computational models rely on having a well-defined system to model. The nature of long-range connectivity of mostly unknown and systems that try to model biologically-realistic models of the specific brain regions require huge supercomputers and several weeks to run, whilst still not being at a level suitable for testing the hypotheses of our project.

Cell culture systems can be useful to determining how one neuron communicates with another, but these systems cannot reproduce the architecture of brain circuits, nor can they incorporate any meaningful model of long-range communication nor interactions with immune systems. Similarly, while brain organoids show some promise in replicating the 3D structure of the brain, they do not yet provide a robust model of brain circuit function and, like cell culture, cannot address our questions relating to rhythmic network activity, long range connections, immune function or cognition. As such, the only suitable model for our research is *in vivo* or *ex vivo* animal 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?

Animal numbers were based on two factors. The first is our extensive experience carrying out this kind of research, which informs our planning process when designing new experiments. We are able to keep animal numbers to a minimum by using cutting edge methods, such as optical recordings of many hundreds of identified brain cells at a time, that yield large amounts of data, and experimental designs that allow multiple measurements to be made from each animal. The second factor was the use of statistical tests that are widely used in both animal and clinical research to allow estimation of the number of animals required to be confident that the results of tests are indeed correct.

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

We estimate effect sizes and adjust required numbers of animals to the minimum required to test our working hypotheses. Furthermore, we employ "within-subject" experimental designs (we measure the same animal before and after a treatment) wherever possible to reduce the number of animals required. Additionally, we maintain ongoing discussions with colleagues about best practice for designing animal experiments and minimising numbers. We take account of ARRIVE (Animal Research: Reporting of In Vivo Experiments) reporting guidelines when designing experiments, which is aided in places via use of the NC3R's Experimental Design Assistant.

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

Beyond good experimental design, we will use a number of other measures to optimise the number of animals used. The majority of the mice used in our project will be for breeding to produce genetically- modified animals for other protocols. We will use best practices, as guided by our animal facility staff, to breed our colonies as efficiently as possible to minimise the number of mice bred, and offer any excess mice to other researchers, when the experimental design allows. We will carry out pilot experiments for new studies to allow an accurate estimation of sample sizes to be made, ensuring that we use sufficient numbers of animals to answer the research question but no more. Finally, when carrying out histological studies, we will retain any unused brain tissue for future use by ourselves or other researchers, ensuring that maximum use is made of the tissue.

Refinement

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

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

The aim of our research is to make inferences about human brain physiology through use of animal models. Mice are one of most commonly used mammalian models in research, and genetically- modified mice allow us to target specific types of brain cell, so mice are the

most appropriate mode for our research as they allow us to gain the largest amount of information from the smallest possible number of mice. Genetically-altered animals will be used wherever possible to model dementia or schizophrenia. The models that we will use are well-validated and are known to faithfully recapitulate specific aspects of human disease, so represent the most refined way of studying these disorders.

The majority (75%) of our experiments will be to study electrical activity in the brain using *ex vivo* brain tissue. These experiments typically involve a surgical procedure to use viruses to insert genes into the brain to allow specific cell types or brain pathways to be studied. By using *ex vivo* preparations, we will minimise pain and suffering to only the days immediately following surgery, after which animals will be deeply anaesthetised before killing for tissue collection. Animals will be housed to the highest welfare standards possible, and all surgical procedures will be carried out using appropriate aseptic technique and post-operative analgesia and care to minimise the suffering and stress.

The remaining experiments will involve immune activation and / or head implants, with or without subsequent behavioural testing. When implanting devices into the brain, we carry these out during the same surgery as any viral injections to minimise the suffering an individual animal undergoes. The immune activation experiments will involve either dietary manipulations, genetic expression of immune molecules, or administration of substances to mimic viral or bacterial infection. In the latter case, we will use well-validated models with the minimum treatment duration required to produce an immune response. We expect that genetic expression of immune molecules or dietary manipulations will provide a more refined and less aversive way of activating the immune system, but these will need to be compared with the models that mimic infection to validate these approaches.

All behavioural tests will be carried out with as little stress to the animals as possible; indeed, many of the behavioural tests promote behaviours that rodents would display in the wild so could be considered a form of environmental enrichment. Some behavioural tests require animals to work for food rewards so in these cases, they will have access to food restricted for a maximum of 16 hours per day. This degree of food restriction is considered mild and animals that undergo this type of restriction have been shown to live longer and be healthier than mice allowed to eat whenever they want.

Why can’t you use animals that are less sentient?
The majority of our experiments (at least 75%) will be gathered from animal tissue or animals under terminal anaesthesia. However, some types of brain activity are linked to different behaviours (such as exploring a maze or sleeping), and understanding how this activity changes in disease is essential for determining which strategies may be effective treatments in human. As such, it is necessary to carry out some experiments in awake, behaving animals.

In terms of life stage, brain circuits begin to form during pregnancy and, in rodents, mature in the first 3 weeks after birth. Molecules used by the immune system also provide important signals for the developing brain. To learn how these systems function in humans, it is necessary to study the interaction between immune and brain systems in these early (neonatal and juvenile) stages. We also need to study the adult stage of development, which is when these systems are fully matured and animals are capable of displaying complex behaviours (such as memory and decision-making) that are directly analogous to those seen in humans. Mice present the ‘simplest’ mammalian organism that have similar nervous and immune systems to humans, so are the least sentient that can be used for our experiments. As such, our project will primarily use mice, and we will only use rats when we need to study neural circuitry or behaviour in an animal with higher

levels of cognitive flexibility than mice are capable of demonstrating (this is particularly relevant for experiments that involve the prefrontal cortex, a brain region important for decision-making and executive function). Finally, dementia is a late-life disease in humans, where progressive memory loss develops after lifetime accumulations of proteins associated with neurodegeneration. It is necessary to used aged mice to reproduce these dementia pathologies in a physiologically-relevant manner.

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

We will monitor new strains of mice very carefully to assess their health and physiology. Post-operative care will include close monitoring of pain and weight, and the use of pain-killers. Animals undergoing procedures that may cause stress will be trained such that they gradually get used to the experimental set-up, spending increasing amounts of time each day with the equipment until it is no longer stressful.

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

We will follow the ARRIVE guidelines throughout.

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

We subscribe to the NC3Rs newsletter and attend their virtual symposia whenever possible. We maintain open dialogue with colleagues in our own institution and beyond to ensure that best practice in animal welfare and experimental designs are maintained.

16. Novel delivery methods for cancer immunotherapy

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, Immunotherapy, Therapeutics, Vaccines, Oncolytic virus

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To develop novel immunotherapy drugs and delivery mechanisms for these drugs which facilitate an improved anti-cancer immune response when compared to currently available 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?

50% of people in the UK (367,000/ year in UK and 17 million worldwide) will suffer from cancer at some point in their lifetime and it will cause 28% of all deaths (166,800/ year in the UK and 10 million/ year worldwide). Immunotherapies have shown that the immune system has the inherent potential to cure metastatic disease even when prior

chemotherapy, radiotherapy and surgery have failed. Currently only a minority, approx. 20% of patients, benefit from immunotherapy due to multiple tumour resistance mechanisms. Research has shown many of the ways in which tumours prevent immune cell attack and there is a great need to develop treatments that can overcome these barriers to improve cancer survival statistics. Prior to human clinical studies only in vivo models can provide the entire immune system in all its complexity for the testing of immunotherapy. During this project the delivery of immunotherapeutic agents to tumours, the magnitude of immune stimulation and anti-tumour efficacy will be investigated as these are all limiting factors for currently used and developing immunotherapies in the clinic.

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

Information for development of therapeutic products and publications including:

treatment regimens to improve delivery of immunotherapy to tumours

efficacy of novel immunotherapy molecules and vectors

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

Information to inform new treatment regimens that will improve delivery of immunotherapy to tumours enabling the most efficacious dosing regimen for all of our immunotherapy projects to be taken forward.

The benefits in the short-term, 2-3 years from first use in the project, will be to shorten the drug discovery process and improve chances of success in formal preclinical studies of new drug candidates by showing their potential efficacy and safety. The information will also be used to guide the design of first in human clinical studies in the medium term, 3-5 years, and eventually as treatment for patients in the long-term, 7-10 years. Delivery of sufficient levels of immunotherapy molecules to tumours has been a limiting factor for efficacy against all carcinomas to date. Publishing peer reviewed articles detailing the efficacy of these treatment regimens will facilitate obtaining further funding to progress towards clinical studies and ultimately to a final therapeutic product in 10 to 15 years time, as well as inform other researchers on the level of efficacy shown in animal models.

Information regarding the efficacy of novel immunotherapy molecules and delivery vectors will lead in the short-term (1-3 years) to go/no-go decisions on each molecule and vector tested. Each molecule has already passed in vitro testing using human cells but is untested in a whole body system similar to that of a cancer patient. These data driven decisions ensure efficient use of time and funding which also reduces the harm to animals and humans by preventing progression to formal preclinical and first in human studies of molecules or vectors that are unlikely to show sufficient efficacy, this is a benefit seen in the medium to long term (4-10 years). The information will also feedback into the in vitro development of our future products ensuring prior to testing in animals only strategies most likely to succeed are taken forward such as immunotherapies targeting particular immune cell subsets (i.e. macrophages, T cells, innate receptors), tumour targets (i.e. particular cell surface targets) and delivery vectors (i.e. RNA, protein or virus delivery vectors). Information may be used to strengthen patent applications that will enable fund raising for products to enter clinical studies. Publishing peer reviewed articles will also assist in fund raising and bring an awareness to the field of effective drug targets.

The longest term benefit (10-20 years) would be to provide information regarding drug interaction with the whole body. Improving the design of immunotherapy molecules and

treatment regimens making it more likely that new drugs developed show efficacy in phase III clinical studies and be medically approved for use improving cancer survival.

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

Outputs will be first maximized by the use of statistical tests and rigorous experimental design to obtain the maximal information for the number of animals used.

The data from the outputs will allow for patent protection and comparison to approved and developing immunotherapy treatments to maximise the chances of obtaining funding to progress to testing in human patients. The publication of data allows for peer review validation of the data assisting fund raising and bringing an awareness to the field of new immunotherapy approaches. During these projects we will be collaborating with academic groups and commercial companies further disseminating knowledge and informing immunotherapy development activities.

Species and numbers of animals expected to be used

- Mice: 3300

Predicted harms

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

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

We will be using adult mice from age 8 weeks up to a maximum of 12 months of age. The majority (>80%) of these mice will be wild-type, but in some instances, they may be genetically modified without a harmful phenotype. Mice are appropriate models as they can be reasonably housed within animal facilities with environmental stimulation and space to socialise and exercise. For our work on the immune system, mice are the least complex animal which still possess a complete set of immune cells with comparable human equivalents. Additionally, cancer and immunotherapy mouse models are the most widely used in the field with a wealth of peer reviewed literature to inform the experiments and limit harm to the animals. Where genetically modified mice are required only already established strains with known phenotypes will be used. These mice will have modifications to components of the immune system or to proteins often expressed in human cancers, to allow growth of implanted murine tumours expressing human proteins and then to test our novel therapeutics i.e., antibodies or viruses.

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

At 8-12 weeks of age, the mice will be administered cancer cells under the skin on the side of the mouse. These cancer cells are derived from common types of cancer such as breast and colon cancer and are routinely used in cancer research. They will be left to grow into palpable size tumours approximately $15mm^3$ in volume. Some groups of mice will then be treated by injection or via oral administration of proteins or chemicals that temporarily inhibit the immune system to help maximise the amount of subsequent immunotherapy that could escape clearance by the immune system and reach tumours. The mice will then be injected intravenously, into the abdomen, or directly into the tumours, immunotherapy in the form of proteins, attenuated virus or RNA. At fixed time

points a blood sample will be taken, for example at day 1 and day 3 after administration of immunotherapy, to measure concentrations of immunotherapy drug in the body. The growth of the tumours will be monitored using callipers or imaging under general anaesthesia at regularly spaced timepoints dependent on the growth rate of the tumours, typically every 1 to 3 days, until they reach a maximum size of 1200mm^3 volume, up to 6 weeks after cancer cell administration. The mice will then be humanely killed and tumours, organs and blood collected for analysis.

In an alternative immunisation protocol where the immune response to a model protein is measured, mice 8-12 weeks old will be administered RNA into the muscle of the leg and the production of a protein measured under anaesthesia using imaging every 1-3 days. The RNA administration will be repeated 1-4 weeks later and at a total of 5-8 weeks the mice will be humanely killed with blood and organs harvested for analysis of immune responses to a model protein.

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

In mice administered tumour cells there should be no measurable effect on the ability to move, eat or drink and therefore no reduction in body weights. If tumours ulcerate and do not heal within 48hrs of developing the mice will be humanely killed. We define ulceration as a full thickness break in the skin and expect this to be a rare event happening in less than 5% of animals. Drug treatments with proteins, chemicals, viruses or RNA may induce a transient reduction of body weight with an acceptable limit of up to 15%. The reduction in body weight is likely to indicate the mouse is in some discomfort and has a loss of appetite similar to a mild infection in humans, it is expected this will not last longer than 48 hours. The use of injections or anaesthesia are only expected to induce low level transient distress like that in humans undergoing vaccination or anaesthesia.

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

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

90% mild, 10% moderate.

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

- Killed

Replacement

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

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

Prior to carrying out animal experiments we have tested all the immunotherapy treatments in vitro using human immune cells and human cancer cells and only those showing an effect are taken forward to test in animal experiments. To measure the effect of different treatment regimens to improve delivery of immunotherapy to tumours an intact circulatory

system, organ pathology and immune system are required. In vitro models such as 2D and 3D co-culture systems which involve mixing a few specific cellular subsets and cancer cells with immunotherapy are used prior to animal experiments, but they cannot completely replace live animal experiments as they do not recapitulate the complexity of the whole body. The whole body modulates the efficacy of immunotherapy through the effects on pharmacokinetics and immunogenicity that cannot be replicated in vitro. Replacement of mice with non-mammalian live animals (e.g. fruit flies) is not possible as the part of the immune system targeted by immunotherapy (the adaptive immune system) is different to that of mammals.

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

In silico methods are used to design our immunotherapy treatments reducing the number of immunotherapy molecules that need to be tested. The literature and databases of immunotherapy molecules that have reported efficacy in previous animal or clinical studies are redesigned to ensure only molecules that are likely to produce results are taken forward to testing, first in vitro, then ex vivo and finally in animals.

Genetically modified reporter cell lines and purified proteins are used in vitro to determine the binding and functional effects of immunotherapy molecules prior to testing in ex vivo models.

Ex vivo models using human peripheral immune cells cultured with human cancer cell lines are already used in our laboratory to pre-screen immunotherapeutic treatments before testing in animals. Those showing no efficacy ex vivo are excluded from further study.

Why were they not suitable?

The immunotherapy treatments to be tested have undergone all the in silico, in vitro and ex vivo testing it is possible to do. Information regarding dosing regimens, pharmacokinetics and immunogenicity can only be obtained in a live animal over a period of weeks after treatment. Stem cells, organoids, ex vivo collected clinical samples cannot replicate the complexity of the whole body required for testing prior to human studies. These non-animal systems compliment and reduce the numbers of animal experiments but cannot currently replace them entirely.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 set a threshold for improvement that is required to show an immunotherapy treatment is sufficiently efficacious based on the literature and 20 years of experience in the field. Statistical significance based on the size of the minimum expected improvement in efficacy and variance is set to be a medium sized biological effect. This shows that groups of 10 mice per treatment are required to be statistically robust.

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

Mice of the same strain and sex will be used for each experiment reducing genetic or physiological differences between animals. Where possible and in the majority of experiments multiple immunotherapy treatments will be simultaneously compared to a single mock treatment group reducing the number of separate experiments required and therefore control groups used.

Randomisation of which animals are assigned to each treatment group will be carried out to ensure an unbiased distribution of animals across groups. Blinding where possible will be used when administering drugs and measuring tumour size to prevent user bias. These techniques decrease variation between groups and prevent the need to repeat experiments due to erroneous results based on user bias.

The use of pilot or dose escalation experiments using a reduced number of animals to determine a maximum tolerated dose or maximal effective dose will be carried out where appropriate. This will allow for fewer treatment groups in the definitive experiment where there are more animals per group as only the effective dose will be tested.

Additionally, where the therapeutics to be tested do not cross-react to murine targets, murine tumours expressing human proteins will be used. In some instances, wild-type mice would reject these tumours reducing statistical power, therefore the use of genetically modified mice or partially immunodeficient mice will ensure successful tumour engraftment and reduce the total number of mice required.
Breeding genetically modified mice is an inherently wasteful process and therefore where possible mice will be bought in from external suppliers as they are required for experiments.

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

Throughout the project, in vitro work testing potential therapeutic treatments and small in vivo pilot experiments will be utilised to reduce the overall number of animals used.

When progressing to in vivo work, the purpose of pilot experiments (typically ~3 animals per group) will be used to identify adverse effects of the protocol and to determine the approximate effect size and effective dose of our treatment. A thorough inspection of the published literature in combination with our past experience will sometimes allow us to accurately estimate dose, effect size, variability and therefore group size, without pilot work. Where this is not the case, data gained from pilot experiments will allow for power calculations to estimate the required number of animals for future work and prevent experiments from having to be repeated because they do not reach statistical significance or use of excessive numbers of animals per group.

Wherever possible when humanely killing animals, we will co-ordinate with other researchers in our facility to share tissues and make any excess material from our mice available to others, thus maximising the scientific output from our work.

Refinement

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

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

Drug treatments: Wherever possible, we will deliver drugs dissolved in the drinking water of the mice. This delivery route avoids restraining the mice and the stress of an injection or a gavage. If this is not possible due to the mechanism of action of the drug, the least harmful appropriate delivery route will be chosen.

Tumour development: The injectable mouse tumour (syngeneic) models that we plan to use have reliable and well characterised disease course, avoiding unexpected increases in severity. When injected subcutaneously or intraperitoneally these tumours rarely metastasize and can therefore be closely monitored. Where murine tumours expressing human proteins or fully human tumours are needed but may be rejected by wild-type mice, genetically modified mice will be used to ensure successful tumour engraftment. Only already available genetically modified mice with known non- harmful phenotypes will be used avoiding immune rejection of implanted cells.

Virus therapy: We will genetically engineer the virus which we use to deliver our therapies to ensure that the virus is attenuated in comparison to wild-type and causes very little harm. This approach has been performed extensively before and is well tolerated by mice.

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

Given that our aim involves utilising the host immune system to target the tumour, we require an animal which possesses a complete set of immune cells comparable to humans. In this context, mice are the least complex animal which still allow us to develop therapies which may translate to humans. While invertebrate organisms such as fruit flies share some components of the human innate immune system, they do not make antibodies or immune memory responses which are a key component of how our therapies will work in cancer. In addition, the vectors which we will use to deliver our therapeutic transgenes virus, protein and RNA have proven abilities to be effective in mice and are well tolerated.

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

We have developed a scoring system for our cancer experiments which implements increased monitoring of the animals as they begin to develop clinical symptoms, ensuring that any welfare issues are detected quickly. This will include implementation of the NC3Rs Mouse Grimace Scale as a sensitive early indicator of distress. Wherever possible, we will deliver drugs dissolved in the drinking water of the mice. This delivery route avoids restraining the mice and the stress of an injection or a gavage.

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

For cancer experiments, we will follow the guidelines set by the National Cancer Research Institute and outlined in Workman et al., British Journal of Cancer (2010) 102, pp 1555–1577. For general administration of drugs, we will follow the LASA Guidelines on Administration of Substances.

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

We will continue to regularly monitor the NC3Rs website for updates relevant to the project and disseminate these updates to all PIL holders. In addition, PIL holders on this licence will be offered the opportunity to attend NC3Rs courses as appropriate.

17. Characterization of novel diagnostic and therapeutic targets in obesity and its associated comorbidities

Project duration

5 years 0 months

Project purpose

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

Key words
Metabolism, Cancer, RNA therapeutics, Fatty liver disease

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We intend to describe how some of the smallest components of cells of the body are involved in obesity and other conditions which exist with it (known as comorbidities) such as type 2 diabetes or fatty liver disease/cancer, with the aim of identifying new potential targets for future 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?

The rising frequency of obesity and its associated comorbidities such as type 2 diabetes (T2D), cardiovascular disease or fatty liver disease constitutes a major health problem. It is estimated that almost 500 million people will suffer from type 2 diabetes worldwide in 2030. In addition, fatty liver disease is becoming an important contributor to liver cancer, mainly hepatocellular carcinoma. Several factors including physical inactivity, aging and poor diet (i.e high calorie diets) are critical contributors to the rising numbers of these

diseases. We need to know more about how the mechanisms linking obesity with liver disease in order to develop new and more effective therapeutic approaches.

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

This project will generate relevant information about how our metabolism in regulated. The results will be published in international journals and diseminated in scientific and general audiences. We will generate a number of new models and research tools.

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

The predicted benefits and beneficiaries are summarized below:

Short term: Metabolism and physiology research. Understanding at the microscopic level how our body copes with daily fluctuations in energy supplies has relevance to how our bodies respond, and to nutrition. The reagents and research data generated in these studies will be a useful resource for the scientific community because it will lay the foundations for future works on metabolism and will allow other scientists to investigate the effect of drugs targeting these mechanisms.

Medium term: Translational research. Our work will use a new therapeutic approach known as RNA therapeutics, what is an emerging area of research. Our studies will evaluate the therapeutic potential of specific substances called 'RNAoligonucleotides'.

Long term: Clinical beneficiaries. The proposed work will develop and test the effect of drugs for the treatment of metabolic disorders such as obesity, fatty liver disease or atherosclerosis (where fatty deposits are laid down on artery walls). Furthermore, the reagents generated could be used to investigate other diseases such as hepatocellular carcinoma (cancer).

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

Research data and reagents generated as a result of the experiments outlined in this document will be made available to the public as quickly as we can. We will disseminate our findings in local, national and international meetings, as well as on our websites, which will be frequently updated and finally we will publish our findings in an open-access fashion.

Species and numbers of animals expected to be used

- Mice: 13910

Predicted harms

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

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

Mice are the lowest vertebrates amenable to genetic manipulations and thus represent an ideal model to study how genes work. For this reason, mice are frequently used to model human metabolic disorders resulting from mutations (changes caused by a range of factors) in specific genes. They also share similar substance-secreting systems to humans

and are susceptible to develop diet-induced diseases such as obesity, which can be equated to humans and thus represent a very useful model to investigate specific metabolic processes. Our experimental models will try to recapitulate the effect of lifestyle factors such as diet in the development of obesity and associated pathologies. For this reason, the experimental models will use adult or juvenile animals.

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

Once we have identified a gene of interest, we will develop methods to modify this gene. Some of these methods consist in the use of viruses or the use specific substances called 'RNA oligonucleotides' to activate or inactivate this gene. Such viruses or substances will be administered to the mice to measure their effects on disease.

Typically, mice with or without a modification in a particular gene will be randomly selected to receive a particular diet or treatment to bring about disease (e.g diet-induced obesity). Initially, we will use a model that recapitulates the initial stages of metabolic pathology such as diet with a high calorie content to promote obesity and mild accumulation of fat in the liver. The diet will be administered for the shortest period to establish the expected symptoms of disease, typically ranging from 4 to 16 weeks. The role of the gene of interest will be investigated by comparison of the development of obesity and other associated diseases in different groups of animals. For this comparison we will monitor changes in body weight, analyse blood samples to check how well the animals process glucose. Typically this is done by simple assays called 'tolerance tests' where we inject (intraperitoneally) a substance such as glucose and monitor how mice are able to process it over a period of 120 minutes. Usually, we perform 1 to 3 tolerance tests over the lifetime of a mouse, and we let them rest at least 1 week between two tests.

Depending on the results, we will use a similar strategy to interrogate the effect of this particular gene in alternative experimental models. In this case we will provide alternative diets or substances to mimic a more advanced stage of metabolic disease such as liver inflammation or fibrosis, but the experimental protocols are similar as described above.

In some circumstances, we will investigate the effect of a particular gene in the development of liver cancer. In this case, we will admisiter by intravenous injection a specific DNA designed to promote the development of liver cancer. We will then inject specific substances such as 'RNA oligonucleotides' to activate or inactivate our gene of interest and evaluate its role in the development of liver cancer.
Tipically, the mice are humanely sacrificed before mice develop any overt clinical manifestations, and effect of the intervention are evaluated by pathological and biochemical analysis of the liver and other tissues.

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

Procedures cause either mild or moderate harm. Taking samples from animals and giving injections causes only brief stress or discomfort. Transplantation of embryos is carried out under anaesthesia and pain relief given where necessary. When a high-fat diet is administered for more than 16 weeks, obese mice may show reduced activity and grooming behaviour and are occasionally prone to skin diseases as a result. Induction of liver cancer is associated with transient distress caused by the injection. In addition, potential side effects included decreased physical activity and weight loss can be observed.

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

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

Moderate 15%

Mild 75%

Subthreshold 10%

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

- Killed

Replacement

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

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

Until now, no computer or laboratory methods have successfully modelled the complex interactions we are studying which occur between parts of the body. For example, some of the effects of hypercaloric diets on the liver can be recapitulated in isolated hepatocytes or ex vivo systems such as liver-on-a- chip. However, some other components such as the reverse cholesterol transport from peripheral tissues to the liver, or the multi-organ crosstalk must be studied in vivo. Therefore, we need to use whole animals.

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

Before performing any work with live animals, we make use of publicly available scientific information. Analysis on the computer helps identify genes of interest and reduces the amount of animal work needed for meaningful results. In addition, much of our experimental work is done in the laboratory using non-animal methods. We are also expanding our non-animal studies to include new methods such as the ‘liver-on-a-chip’ to mimic the diseases we study.

Why were they not suitable?

As the complex systems we study involve different organs of the body, we do need to include animal studies in our research, although we use non-animal systems mentioned previously wherever possible. We promote the 3Rs by using these models.

Reduction

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

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

The numbers have been estimated based on power calculations, our previous experience and similar studies published in the literature in order to achieve meaningful results. We estimate 1250 mice for each of the experimental protocols, 10000 animals for breeding purposes and 160 mice in the event of generating novel genetically modified mice.

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

The experimental design is made considering the NC3Rs Experimental Design Assistant and taking advice from local biostatisticians where necessary.

At every stage in our experiments, consideration will be given to ways in which we can reduce the number of animals. Several of the protocols that we use are designed in such a way to obtain the maximum possible data from a single animal. For example, various organs are dissected from an experimental mouse or we take multiple samples from a single piece of animal tissue.

The generation and maintenance of genetically modified mice considerably increases the number of mice used in a research project. For this reason, and whenever possible, some of the procedures needed to alter genes will be carried out in the laboratory rather directly on the animals themselves, thereby significantly reducing the number of mice involved.

If this is not possible we will first check if animals with the genetic make-up that we need already exist by sending notices via the ‘mouse locator’ services and searching mouse databases and publications.

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

Efficient breeding is assured with assistance from experienced staff within our animal research facility. Where possible we will use strategies that maximise the use of offspring when breeding genetically modified mice. In addition, we will ensure that new-born litters are not disturbed to maximise the survival of the offspring.

We will ensure that the use of tissues and samples from each experiment is maximised to obtain maximum information from each mouse. Spare animals will be made available for use to other scientific projects.

When appropriate, we will freeze mouse lines that are not required for extended periods, rather than maintaining stocks.

Refinement

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

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

Where possible, genetic changes to modify the expression of a gene of interest, will be made to specific parts of the mouse rather than to the whole animal to lessen potential adverse effects associated with whole-body genetic changes. This will also improve the quality of our research findings.

To administer specific diets or drug treatments, we will use the lower dose and during the shortest period of time possible to obtain meaningful results. When medium-long term treatment is needed, wherever possible these will be delivered using pellets rather than by giving the animals multiple injections. In addition, to prevent pain whenever is expected pain relief will be provided.

When using a model of liver cancer, we will use the hydrodynamic tail vein injection model, instead of other models (e.g DEN injection, of streptozotocyn plus western diet), as it produces meaninful results in a shorter period of time, reducing unnecesary suffering of the animals.

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

Our studies are generally performed in adult mice. Anaesthesia has a strong effect on metabolic rate and it is therefore not usually possible to study metabolism in anesthetised animals, however, when possible, general or terminal anaesthesia will be used (e.g local anesthesia in i.v injections) to minimise stress and suffering of the animals.

Many key metabolic processes are not conserved in lower organisms such as non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) or less sentient animals (eg zebrafish). Then these models cannot be used as alternatives for our studies.

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

When viral vectors are being used, wherever possible, adenoviruses will be replaced by adeno- associated viruses which trigger a much lower immune response. Animals will be kept warm and monitored regularly during and after anaesthesia. Sterile methods will be used and pain relief will be given as necessary. Any animal experiencing undue distress or which has reached the defined limits pre-agreed in this licence will be humanely killed. We will regularly consult with CBS staff, vets and colleagues about best practice and potential further refinement of our procedures.

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

We will keep following recommendations from the local 3Rs advisory group, Laboratory Animal Science Association (LASA), Animal Research: Reporting of In Vivo Experiments (ARRIVE) and governmental documents such as the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. In addition, we follow in daily basis scientific publications and will specifically look for improvements in our animal work protocols which could lead to a refinement in our experimental work.

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

I will maintain regular conversations with some members of the local 3Rs advisory group and other colleagues regarding 3Rs and other improvements of our animal work. Databases such as the NC3R's mouse database (http://nc3rs.org.uk/GAmice) will be consulted regularly.

18. Host-pathogen interactions in the immune system and CNS

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

immune system, CNS, infection, prions, ageing

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to determine how infectious microorganisms "hijack" or exploit the body’s immune system to establish infection, and in some cases spread to the brain where they can cause damage to neurons. In addition, the second major aim is to understand how additional factors such as ageing, inflammation and co-infection with other pathogens can affect the function of the immune system, and susceptibility to 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.

Why is it important to undertake this work?

One of the main pathogens that will be studied in this project is prions which affect humans and animals. Infection with prions causes extensive neurodegeneration in brain ultimately leading to death. Prion diseases are currently untreatable. If we can understand the mechanisms that prions use to establish infection, we may be able to design novel therapeutic strategies to block these currently untreatable diseases. Over 26% of the UK population is >65 years old and this is expected to rise significantly in future decades.

Immunity in the elderly is significantly compromised by ageing and is associated reduced vaccine efficacy and increased incidence of infectious diseases and cancer. A thorough analysis of the factors that underpin the dramatic ageing-related decline in immune function will help identify the ageing-related factors that influence pathogen susceptibility and aid the development novel approaches to improve immunity in the elderly.

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

Identification of novel cellular and molecular mechanisms that influence disease pathogenesis and susceptibility to prions and other important pathogens, especially gastrointestinal pathogens. The identification of new cellular and molecular mechanisms could identify new therapeutic targets to help to protect against infection.

An improved understanding of how inflammation and ageing affect the development and function of the immune system, and how this can affect the immune system's ability to protect against pathogen infection.

A detailed analysis of how concurrent pathogen infections can influence prion disease pathogenesis and susceptibility to other infections.

Data from this project will be deposited in publicly-accessible databases. Where appropriate, useful publicly available web resources cataloguing the range of genes expressed by important immune cell populations will also be created.

Dissemination of findings and data related to the above outputs through open-access scientific publications, presentations at scientific conferences, seminars to relevant stakeholders, creation of public engagement activities to describe the outputs of this project to the general public and stakeholders, and where appropriate engagement with the media.

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

In the short term the international immunology, mucosal immunology and neuroimmunology scientific communities and other interest stakeholders will gain information on the role of how specific cells in the immune system are exploited by pathogens to establish infection. They will also gain an understanding of how additional factors such as old age, inflammation etc. can affect the function of these cells, and how this affects susceptibility to pathogen infection.

Scientists and industrial stakeholders (eg: pharma/biotech. companies) may benefit from data on cellular/molecular mechanisms that regulate the immune system and its ability to provide protection to certain infections. In the longer term, this could lead to the development of novel treatments to prevent or treat these infections.
Our collaborators and other interested scientists will also benefit through having access to data and unique biological samples generated from this project. Many other scientists may also benefit from access to these samples or data derived from them.

UK policy makers will have interest in the project's findings on factors that may influence prion disease susceptibility. This may influence their assessments of the risk of prion disease transmission.
How will you look to maximise the outputs of this work?

Data from the experiments described in this report will be disseminated to the international

scientific community through a combination of publication in peer-reviewed journals, data deposition in public databases and presentation at scientific meetings. Studies will be submitted whether the data describe significant/exciting advances, or conversely, show no effect of treatment etc. The latter are equally as important, and by publishing these so-called "negative-results" studies this can help to reduce the number of animals used in future studies by preventing unnecessary experiments being undertaken.

Where possible, opportunities will be taken to communicate the project's findings and implications to the public, through a range of public-engagement activities. The applicant's organisation has a dedicated and very active public-engagement department and helps the scientists to design a range of activities to explain the importance and impact of their research to a range of audiences from schools, to public open days, targeted stakeholder groups, and larger public agricultural events and scientific shows/discussions.

The release of potential news-worthy publications will be discussed with the Institute's press officer and press-releases issued when appropriate. The applicant has much experience of this. Many of the applicant's publications from recent project licences were handled in this manner and attracted much media interest, including The Times newspaper and BBC news. The applicant has also given interviews to organisations such as "Understanding Animal Research" to help explain to lay-audiences the important and useful findings obtained from animal studies undertaken in the lab.

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.

The aims of this study are to determine how pathogens such as prions exploit cells and tissues of the immune system to spread from the site of exposure (eg: intestine), establish host infection and ultimately cause disease in the CNS. We are also interested in how factors such as ageing, inflammation, pathogen co-infection and the presence of the "friendly" commensal microbiota can affect affect development of the mucosal immune and central nervous systems and how these affect susceptibility to infections. These cannot be achieved using species outside the animal kingdom which do not have complex digestive, immune and nervous systems. The complex interactions between these systems is also difficult to study in reduced in vitro systems.

For these reasons it is essential to use mice, and young-adult/adult/aged mice will be used throughout. Among mammalian model organisms, the information and literature on basic anatomy and physiology of the immune and central nervous systems is most extensive for mice which provides the best platform for understanding data produced in this project in the context of previous work. Although differences do clearly exist between species, the major features of the immune system are conserved in mice and humans allowing meaningful extrapolation. Indeed, the findings from this project will be directly relevant to enhancing our understanding of disease pathogenesis and susceptibility in large animal species (sheep, cattle, deer etc.) and of course, humans.

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

The animals used in this project may undergo one or more of the following procedures, for example: undergo surgery, irradiation followed by reconstitution with haematopoietic cells such as bone marrow, injections of substances, and infections with some pathogens. Some animals will experience only one of these, but in some studies these may be used in combination depending on the nature of the study. Experimental duration will range from acute (hours), to days (less than 1 week), to several weeks to months, depending on the study.

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

Some of the mice in this study will be infected with prions or other infectious agent and a proportion of these will develop clinical disease: moderate severity. However, to avoid unnecessary suffering these mice will be culled at the onset of definite clinical signs. The duration of oral prion infection in mice is very long ~340 days. However, for approx. 90% of this period the mice are free of clinical signs and appear healthy. In many studies the majority of the mice will be humanely culled during the pre-clinical period before they show signs of clinical disease.

Other mice may also undergo periods of moderate severity, for example after a short exposure to irradiation. A split dose of irradiation is used to reduce the impact of the adverse effects on the mice. However, in this instance the period of severity and weight loss is transient because soon after irradiation the mice are given a bone marrow transplant. Following bone marrow transplant their body condition and weight typically recovers within a few days of treatment.

Where necessary and appropriate analgesia may be given to reduce 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)?

Many of the mice will experience no greater than mild severity after the procedure. However, some animals (approx. 10%), such as those that undergo irradiation followed by reconstitution with haematopoietic cells such as bone marrow may experience a transient period of moderate severity. Similarly, some animals may develop moderate clinical signs of infection. However, not all of the infected animals will be maintained until they do develop clinical signs. The majority (approx. 60+%) will be culled before they develop detectable clinical signs and tissues analysed ex vivo.

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 aims of this study are to determine how pathogens such as prions exploit cells and tissues of the immune system to spread from the site of exposure (eg: intestine), establish host infection and ultimately cause disease in the CNS. We are also interested in how factors such as ageing and the commensal microbiota affect development of the mucosal immune and central nervous systems.
Experimental systems that allow this are limited and must involve the use of vertebrate animals. Therefore, this cannot be achieved using species outside the animal kingdom which do not have complex digestive, immune and nervous systems. The complex interactions between these systems is also difficult to study in reduced in vitro systems.

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

Every opportunity will be taken to exploit computational methods during the study these are inadequate on their own because the complexity of interactions between the nervous and immune systems. Where possible we will also use data available in published transcriptomics studies, and have done so in many recent studies. These negate the need to use additional sets of animals to obtain cell/tissue preparations to generate our own samples.

Organoids and/or intestinal enteroid cultures: Wherever possible this project will also in vitro systems of isolated cell populations (some derived from cell lines not requiring the use of animals) to address specific questions or to complement the in vivo studies. Many in vitro studies of pathogen uptake across the gut epithelium have typically used monolayers of apparently differentiated enterocyte cell lines. However, it is questionable whether these systems are accurate representations of the gut mucosa as they lack the complexity of the mixture of cell populations in the gut epithelium. A recently described 3D, physiologically relevant in vitro model of the gut epithelium provides an alternative whereby gut organoids (enteroids) are established in vitro from isolated intestinal crypts in a collagen-based matrix. These undergo multiple crypt fission events, generating many villus-like epithelial domains surrounding a central, closed lumen. These enteroids maintain the cellular diversity that is present in the gut epithelium (enterocytes, goblet cells etc.). They can also be maintained long term enabling the chronological in vitro study of host-parasite interactions. The isolation and propagation of enteroids is well established in the applicant’s lab. Importantly, these have been used in his lab. to study the role of CSF1R-signalling in maintenance of intestinal crypts, and have also been developed from bovine, porcine and avian tissues and have been used to study aspects of host-pathogen interactions. A particular benefit from the use of these enteroids is that they can be maintained for weeks and serially passaged. Thus, many 100’s of enteroids can be obtained from a single tissue sample (~20 cm of intestine) negating the requirement for large numbers of animals. Thus, where possible, enteroids may be used as an in vitro alternative to provide further characterisation of data derived from in vivo studies. Where appropriate these enteroids may also be used to test hypotheses before subsequent development of in vivo studies. This approach was successfully used to study the effects of Salmonella Typhimurium on gut epithelial cells. Alternatively, data obtained on the effects of ageing on intestinal crypts was investigated further using mouse enteroids to identify potential molecular mechanisms.

Detection of prions: At present there are no other reliable methods that enable prion agent infectivity levels in cells or tissues to be accurately estimated other than by recipient

mouse bioassay. The magnitude of prion disease specific PrP^{Sc} protein (a major if not sole component of infectious prion particles) can be used as a reliable surrogate. The relative level of PrP^{Sc}-seeding (converting) activity can also be a useful comparison and will be used where appropriate. A tissue culture-based prion agent infectivity bioassay has been described with potentially promising results. However, there are limitations with such assays, not least sensitivity.

Why were they not suitable?

As mentioned above, some of the listed techniques can be very useful and can be used to replace/reduce the need for live animal studies. However, as described below these do have some limitations.

While every opportunity will be taken to exploit computational methods during the study these are inadequate on their own because the complexity of interactions between the nervous and immune systems. The lack of existing data upon which to construct accurate models in silico introduces too many uncertainties that would compromise data interpretation. However, the applicant regularly exploits publicly available data bases to obtain useful data on the effects of certain treatments, immunodeficiencies etc. on gene expression data.

At present there are no other reliable methods that enable prion agent infectivity levels in a wide range of cells or tissues to be accurately estimated other than by recipient mouse bioassay. Accumulation of the disease-specific isomer of the prion protein, PrP^{Sc}, can provide an estimate of the magnitude of prion infectivity in diseased tissues. However, immunoblot detection of PrP^{Sc} is less sensitive than mouse bioassay of infectivity, and PrP^{Sc} and infectivity can sometimes be separated in vitro and in vitro. The applicant's research has also demonstrated that during the very early stages of a prion infection, detectable levels of PrP^{Sc} in lymphoid tissues can often lag behind that of prion infectivity. PrP^{Sc}-seeding activity assays: This is very sensitive at detecting the ability of PrP^{Sc} in a sample of interest to convert normal cellular PrP^{C} into the prion disease-specific isoform. Although useful, these assays do not directly detect prion agent infectivity.

Cell culture based prion infectivity assays: However, there are limitations to using this assay since its reliability appears to be restricted to a particular prion agent isolate (RML scrapie prions) that is not used at our Institute. The vast majority of the data we have obtained on the pathogenesis of prion disease over the past 25 years has been obtained using the ME7 scrapie agent strain. This mouse- passaged strain was originally derived from a sheep with natural scrapie and our data suggests provides an excellent mouse model to study prion disease pathogenesis in larger species. The pathogenesis appears to be similar to natural sheep scrapie, CWD in cervids (deer, elk etc.) and vCJD in humans. The RML prion isolate, in contrast is not a strain and instead is a mixture of 2 agents which can vary in pathogenesis depending on the host. This obviously questions its use in in vivo pathogenesis studies as it is difficult so separate the prion effects from the host effects, and confidently conclude which are the important factors. Thus although a cellular assay can be used to detect RML prions, we do not consider it appropriate to use in the current project. Furthermore, we have no experience of using the RML prion isolate and would need repeat many of our past experiments to obtain baseline 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?

Good principles of experimental design will be applied to ensure the minimum numbers of animals necessary to achieve satisfactory results are used. All experiments are critically reviewed by the named vet, NACWO, NTCO and a statistician prior to commencing. A study request is submitted which includes a description of the objective and hypothesis, experimental groups, treatments, and assessment of effects, methods of data collection and statistical analysis. Formal procedures are in place within our Institute for review and approval of each experimental study prior to their commencement. This review system is intended to ensure that the numbers of animals utilized is at a minimum whilst ensuring that robust and meaningful results are produced for each experiment. No study involving the use of experimental animals can be undertaken in the Institute without having successfully undergone this critical review.

The numbers of animals allocated to each experimental group is reviewed continually for each experiment and judgements made based on previous experience. Professional statistical advice will be sought for complex study designs– this is available through the Institute's scientific and ethical review committee, which includes a qualified statistician. This protocol is designed to ensure that numbers of experimental animals are kept to a minimum, whilst at the same time ensuring enough animals are used to produce statistically significant results. The applicant's experience of >25 years of working with the pathogen infection models described in this project has shown that groups between 6-12 mice are required to give reproducible and statistically significant incubation periods following inoculation by peripheral routes. The minimum statistically useful numbers of animals to use for serial kill time points is 4 animals, and has proved reliable in recent studies. These group sizes will be used unless there is sufficient justification for including a larger number of animals, for example when first analysing the influence of a factor for which we have limited previous experience. Previous experience has shown that groups of 4-6 intracerebrally injected mice are sufficient as controls to eliminate the possibility that prion disease pathogenesis is affected once disease is established within the brain. As the experiments in this project licence progress, further experience will be gained from the use of these experimental systems and group sizes revised accordingly. Indeed, throughout the duration of this licence, group sizes in such studies will continue to be assessed and refined, where considered appropriate.

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

In addition to the measures described in the above section, the inclusion of the use of these transgenic mice in prion infectivity bioassay experiments has reduced both the number animals required and their duration in experiments.

To attempt to reduce the numbers of animals used in prion agent infectivity bioassay experiments, the use of genetically altered PrP-overexpressing Tga20 mice was explored. As these mice develop prion disease with much shorter incubation periods than wild-type mice, the duration of these experiments is substantially reduced (from typically ~300 d to

~130 d). The savings encountered through use of Tga20 mice include the following (using the ME7 scrapie strain):

-Much smaller group sizes. Use of 4 recipient Tga20 mice is sufficient to provide statistically useful data in bioassay experiments, compared to at least 6 wild-type mice.

-Reduced typical prion disease incubation period from 300 to 120 days.

-Reduction of maximum experiment duration from 500 days to no more than 200 days.

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

Many of the typical measures used have been described in the above sections. However, we also will implement efficient breeding procedures. We have also found that targeted pilot studies aimed at determining for example, the optimal dosing regimen can provide a significant saving. These have helped to eliminate the need for much larger titration experiments; have identified the optimal duration of dosing or effect; identified treatments/protocols that return a null result. The latter has prevented the unnecessary use of much larger numbers/groups of animals in a large experiment that would have returned a negative result with no additional scientific benefit. This has also helped to identify potential unexpected adverse reactions.

Refinement

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

Which animal models and methods 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 C57Bl/6 mouse strain will be used in many experiments (either as wild-type or as a background strain for the genetically-altered animals, GAA). Our data show that the C57Bl/6 strain develops age- related changes similar to the immune system similar to those observed in large animal species and humans. We will use wild-type mice (Eg: C57Bl/6J) and genetically altered mice without harmful phenotypes as animal models. As a consequence, these animals in their unperturbed states are not expected to experience harmful effects as a consequence of the genetic alteration.

Key approaches in this project include the use of animals with specific immunological modifications to enable the manipulation of the immune system or sub-populations of cells. In mammals, such technologies and associated reagents are currently most widely available for mice. purification of immune cells for ex vivo analysis (gene expression profiling by mRNA-seq) and live imaging of specific cell populations (e.g. mononuclear phagocytes). Each of these can only be achieved by using animals where these cell populations are easily identified. Currently, transgenic mice expressing fluorescent labels such as GFP offer the only practicable means to identify specific cell types for these applications. Mice ideally suited to fulfil the objectives in the project are available within the Institute (e.g. CSF1R- EGFP-tg mice which express GFP selectively in mononuclear

phagocytes).

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

The in vivo experiments will use mice. A mammalian species is necessary since the main aim is to investigate interactions between certain pathogens and host immune cells and tissues, including those in the CNS. As a consequence, it is not possible to examine this in less sentient species.

Data generated from this project will be directly relevant for enhancing our understanding of disease pathogenesis and susceptibility in large animal species (sheep, cattle, cervids etc.) and humans.

Among mammalian model organisms, the information and literature on basic anatomy and physiology of the immune and central nervous systems is most extensive for mice which provides the best platform for understanding data produced in this project in the context of previous work. Although differences do clearly exist between species, the major features of the immune system are conserved in mice and humans allowing meaningful extrapolation.

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

Any of studies involving surgical or invasive intervention will adopt appropriate pain management and post-operative care.

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

The ARRIVE (Animal Research: Reporting of In Vivo Experiments; arriveguideline.org) guidelines will be used to ensure experiments are designed and conducted in the best possible manner to address the principles of the 3Rs, and to also ensure they are reported in sufficient detail enable maximal reliability and reproducibility.

The PREPARE guideline (https://norecopa.no/prepare) cover the three broad areas which determine the quality of the preparation for animal studies: 1, Formulation of the study ; 2, Dialogue between scientists and the animal facility; 3, Methods.

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

We will stay informed about the advances in the 3Rs by attending informational events provided locally, obtaining information from our departmental website and through regular communication from our named information officer (NIO). Information on advances on the 3Rs will be provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and through other sources such as the Norecopa newsletter and the PREPARE guidelines.

19. The neural control of movement in health and disease

Project duration

5 years 0 months

Project purpose

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

Key words

Spinal cord, Brainstem, Motor Neuron Disease, Movement, Spinal cord injury

Animal types	Life stages
Mice	neonate, juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The first, basic aim of this project is to advance our understanding of the neural networks within the brainstem and spinal cord, which control movements such as breathing and walking (locomotion). The second, more translational aim of this project is to reveal disease mechanisms underlying the devastating, fatal neurodegenerative disease known as Motor Neuron Disease (MND) or Amyotrophic Lateral Sclerosis (ALS).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 treat a range of diseases and injuries that alter normal movements or cause paralysis by damaging parts of nervous system, it is critical that we reach a much better understanding of how these systems work in their normal, healthy state. We must then use

this 'basic' knowledge to guide parallel research that determines what changes occur within these motor control regions during disease or following injury. By performing such complementary strands of basic and translational research, we will be ideally placed to reveal new targets and ultimately develop new treatments for devastating, often fatal, diseases and injuries that prevent normal movements. In the present project we will focus on motor control regions within the brainstem and spinal cord, which control breathing and walking. We will study how neural networks in these regions control movements in their normal, healthy state. We will also investigate how these neural networks change in models of Motor Neuron Disease (MND), and in doing so reveal novel therapeutic targets for this devastating and incurable disease.

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

We expect to generate a significant amount of new information regarding components of neural circuits that are critical for the control of movements, in particular walking and breathing. In addition, we expect to generate important new knowledge about changes within these neural circuits that contribute to dysfunction and neuronal loss in Motor Neuron Disease. This output will most likely take the form of open access research publications in international, peer-reviewed journals.

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

The most likely short-term beneficiaries of the outputs from this project will be members of the wider academic community. We expect our findings to be of significant interest to thousands of researchers worldwide, including many involved in research that goes beyond the immediate field of motor control. Our findings are likely to help direct and form the basis for many subsequent studies aiming to advance our fundamental understanding of how neural networks control behaviours and how neural network dysfunction leads to disease. In the longer-term we expect our outputs describing changes to neural networks in Motor Neuron Disease (MND) (MND) models to be utilised by biotech and pharmaceutical companies to inform drug discovery and development programs, which will ultimately benefit patients. Recent evidence of this includes a collaboration we have formed with a biotech company to assess whether drugs that target communication between glial cells and neurons represent viable candidates for novel MND therapeutics. MND is a devastating, uniformly fatal condition, for which the risk of diagnosis is 1 in 300. MND symptoms typically occur at mid-life and the average life expectancy from the point of diagnosis is less than 3 years. There remains no cure for MND and current treatments only extend life by 3-4 months. Thus, there is a desperate need for new knowledge that will lead to novel therapeutic strategies, which aim to treat and eventually cure MND.

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

We will maximise the outputs of our work by sharing our findings rapidly and widely. We will disseminate new knowledge promptly at national and international conferences and subsequently via publication in leading international, peer-reviewed journals. We aim to publish all our work in open access formats, so that it reaches the widest possible audience, including non-specialist stakeholders. Rapid and wide dissemination will facilitate the establishment of new collaborations with research scientists across multiple fields and disciplines, as well as industrial partners. We will also continue to share our findings with the broader public, including people affected by MND, via social media platforms, public outreach events, public lectures and, where applicable, press releases.
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.

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

In order to investigate and understand the complex neuronal networks of mammals, and how they are affected by disease, it is necessary to study central nervous system tissue obtained from them. We have chosen mice, typically utilised at early postnatal stages, as our animal model because this allows is to perform carefully controlled experiments on isolated (*ex vivo*) spinal cord and brainstem tissue, without the need for whole animal (*in vivo*) experiments. Mice have also been chosen because of the availability of a growing number of genetic techniques, which allow labelling and manipulation of specific cells within the nervous system and the insertion of mutant genes that cause human diseases.

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

The majority of animals (~75% of an estimated total of up to 12000 over 5 years) will be used as part of a breeding and maintenance programme to generate specific types of genetically altered mice for experiments. As part of this programme, small biopsies will need to be taken from animals to determine what genes they carry. A subset of animals (~25%), which are born with the correct combination of genes will be used for *ex vivo* experiments. Prior to these experiments some animals (~5% of all animals used on project) will be injected with substances to label specific subsets of cells within the nervous system. Injections will be performed by the least invasive method possible. This will either be via injection into the body cavity (intraperitoneal), injection between the chest wall and lungs (intrapleural), or injection directly into specific muscles (intramuscular). Intrapleural and intramuscular injections will be performed under general anaesthesia. Animals will then be killed via the most humane method appropriate and central nervous system tissue removed for use during *ex vivo* experiments.

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

The genetic alterations present in most animals are expected to have no impact or adverse effects. A subset of genetically altered animals used to model human disease may develop motor deficits, such as shaking of limbs (tremors), abnormal walking patterns (gait abnormalities) or hyperactivity leading to a reduction in weight gain during adulthood. Animals are also likely to experience brief, mild pain when tissue biopsies are taken for the purposes of determining which genes they carry and when intraperitoneal injections are performed. Some animals may also experience up to moderate pain associated with intrapleural or intramuscular injections following these surgical procedures and therefore require administration of pain killers (analgesics).

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

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

Based on our previous project licences, we expect the severity of the procedures conducted on the majority (~65%) of animals to be classified as sub-threshold. We expect the severity experienced by some animals (~30%) to be classified as mild. Finally, we expect the severity experienced by a smaller subset of animals (≤5%) to reach the classification of moderate.

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

- Killed

Replacement

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

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

In order to study and fully understand the complex neuronal networks of mammals, including how specific components of these networks ensure appropriate output is produced for the control of movement and what goes wrong within these networks during disease states, it is necessary to study central nervous system tissue obtained from them.

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

We plan to continue to utilise human stem-cell based models, human post-mortem tissue and some computer-based modelling in parallel with mouse tissue.

Why were they not suitable?

Human stem-cell derived cell culture models cannot fully replace the use of animal tissue because cultured cells cannot recreate the complex networks we seek to understand. Post-mortem tissue only provides a ‘snapshot’ of the end of disease and cannot be utilised for functional analyses. There also remain too many unknowns for computer simulations to fully replace the use of animal tissue.

Reduction

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

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

The number of animals to be used in the proposed project has been estimated from the published reports of other researchers using similar techniques to answer equivalent research questions and from the ~15 years of similar research conducted in my laboratory under my previous project licences.

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

Where statistical analyses are to be utilised we first determine the minimum number of animals required to obtain significant results. Importantly, this takes into account the likely breeding success, predicted number of animals carrying the required genetic alterations and the expected success rate of experiments (e.g. how many cells we typically record from within each piece of isolated tissue). 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 *ex vivo* preparations, which last up to (and sometimes more than) 6 hours allowing data to be collected from many spinal and brainstem neurons per preparation. Animal numbers will also be reduced due to our planned use of state-of-the-art genetic techniques. It has historically been difficult to define specific subtypes of spinal and brainstem neurons for subsequent study. This has meant that researchers have had to record from a large number of neurons, even though they have been attempting to study a small, specific population of neurons. In the present study we will utilise the most recent genetic techniques available to label and manipulate discrete populations of spinal and brainstem neurons. Since we will be able to identify subtypes of neurons prior to establishing recordings, we will be able to reduce the number of recordings, and hence animals, required to meet our objectives.

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

We will continually optimise breeding strategies to produce the maximum number of animals with the required genetic alterations. As a consequence of using genetic techniques in which animals need to carry multiple inserted genes, a number of the offspring generated will be unsuitable for experimental purposes. Some of these animals will, however, be utilised for future breeding or as control animals. Where appropriate, pilot studies will be used to determine the variability in experimental outcomes and therefore the total number of animals needed to provide valid results. We will continue to optimise each set of experiments to maximise the amount of data obtained from each animal. For example, when investigating the properties of specific types of cells, individual cells are the experimental unit, so we will optimise conditions (e.g. tissue viability and duration of recordings) to allow for the greatest number of individual cell recordings possible from each piece of isolated tissue. We will also continue to share tissue (e.g. other organs such as the heart) from animals we have killed with other researchers when appropriate.

Refinement

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

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

We will utilise mice, because of the availability of genetically altered animals that allow us to address our main aims and objectives. For our fundamental studies, the genetic

alterations used, which will allow specific populations of spinal neurons to be labelled and manipulated, should have no harmful effects on the animals. For our translational work, animals will carry mutant genes that cause Motor Neuron Disease-like symptoms. These symptoms may include tremors, abnormal walking patterns and, eventually, muscle weakness leading to progressive paralysis. However, because we aim to determine early disease mechanisms, we will carefully monitor disease model mice and use them for breeding, experimental purposes, or kill them using humane methods prior to the onset of significant suffering due to disease.

Alongside genetic techniques, we will also utilise retrograde labelling techniques to label specific types of cells in the spinal cord and brainstem. Our default method for retrogradely labelling cells will be via the injection of substances into the body cavity (intraperitoneal). This will reduce the need for more complex surgical procedures. However, for some experiments, such as those examining neural properties that contribute to the selective vulnerability of different subtypes of motoneurons in Motor Neuron Disease, substances will be injected into muscles (intramuscular) or a space between the chest wall and lungs (intrapleural) to label more specific neuronal subtypes than is possible via intraperitoneal injection.

The choice of mice as the animal model in the present study also importantly allows us to perform analyses in isolated (*ex vivo*) spinal cord and brainstem tissue. By using acute *ex vivo* preparations we can investigate complex neuronal networks whilst avoiding the pain and suffering associated with *in vivo* experiments involving whole animals. Following the breeding of genetically altered mice and/or injection of substances to label specific cells, animals will be used in acute anatomical or physiological experiments, providing a clear endpoint and minimising suffering.

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

The use of isolated mouse tissue is necessary to allow us to study the complex networks of mammalian neurons and produce new knowledge that can ultimately be applied to human health and disease. We cannot utilise more immature life stages because the neural networks we wish to investigate are not sufficiently formed until postnatal stages. For one of our protocols where anaesthesia is used a period of recovery will be required before animals are killed and tissue extracted for analyses. This is necessary to allow time for the injected substances to travel into the nervous system and label specific cells.

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

The majority of animals will experience no harms associated with the genetic alterations they harbour and will be killed via humane methods prior to extraction of tissue. We will minimise the number of animals that require additional procedures (injection of substances) by seeking out and utilising non- harmful genetic approaches where possible. Where surgery is required to inject substances, we will use appropriate peri-operative care measures, as advised by the NVS, and surgeries will be carried out under aseptic conditions. When using genetically altered animals to model disease, we will perform regular and careful monitoring to ensure that animals are killed prior to significant suffering. Such monitoring will be performed more regularly for any new types of genetically altered animals that we do not have previous experience with and for which detailed data may be lacking.

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

The *ex vivo* experimental techniques we will perform using isolated nervous system tissue have been refined by many laboratories around the world to improve viability and reliability of the preparations. We will continue to follow the latest scientific publications that describe the most relevant experimental methods utilising isolated nervous system tissue to ensure we maximise the likely success of our *ex vivo* anatomical and physiological studies. In addition, we will utilise relevant guidance published the Joint Working Group on Refinement.

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

We will continue to discuss possible improvements to our methods with our excellent team of animal care technicians. I will also benefit from my on-going membership on the University's Animal Welfare and Ethics Committee (AWEC), where advances in the 3Rs are routinely discussed. In addition, I will obtain ideas for improvement via fora associated with organisations such as LASA and the NC3Rs.

20. Dynamics of stress axis physiology in health & disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stress, Pituitary, glucocorticoid, corticotroph, physiology

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to understand how stress is regulated by investigating the dynamic control of anterior pituitary corticotroph physiology and stress axis function in health and disease. Understanding these mechanisms is fundamental to our ability to diagnose and ultimately treat major human stress-related disorders such as obesity, depression and hypertension.

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

Why is it important to undertake this work?

Stress and stress-related illness has a major impact on the UK economy accounting for > 40% of all working days lost with an estimated annual cost of £6billion with additional burden through effects on animal health, welfare and food production. Understanding the mechanisms controlling the stress response is important for our ability to diagnose and ultimately treat stress-related disorders.

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

This project will generate a number of outputs of benefit to the scientific community, policy makers and general public including:

New information on the physiology of the stress axis disseminated through peer reviewed publications in open access journals as well as through conferences, seminars and other scientific outlets.

New resources ranging from databases of gene expression, new animal models and other novel tools/resources (e.g. antibodies) and predictive computational models that will be made freely available to the scientific community.

New insights and recommendations for clinicians and policy makers for the diagnosis and treatment of stress-related disorders

Materials and resources for wider public understanding of stress and physiology through public engagement and outreach activities

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

Shorter term benefits (0-5 years)

These are predominantly to the basic science community that will provide a mechanistic framework for understanding three important areas in lifelong wellbeing and disease: i) the mechanisms controlling physiology of anterior pituitary corticotroph cells that control the stress response, ii) how corticotroph cell heterogeneity and network population dynamics control systems level behaviour iii) new insights into the control and recovery of the hypothalamic-pituitary-adrenal (HPA) stress response in health & disease and identification of new diagnostic and therapeutic approaches. Importantly, the fundamental importance of understanding these processes is essential to communicate to policymakers and general public that will be facilitated through outreach, public engagement and policy engagement forums.

Longer term (5 yrs +)

In the longer term the work will inform translational and applied human and animal research as well as providing possible new diagnostic and therapeutic strategies to treat stress-related disorders that could have significant implications for both human and animal wellbeing and welfare and impact on UK economic prosperity. For example, glucocorticoids are one of the most widely prescribed drugs and stress related disorders result in >£6billion burden to the UK economy including through absence from work and our studies include collaboration with clinicians. Thus understanding how the stress response is regulated and can be manipulated holds the promise of modifying both current practice in how stress is managed and glucocorticoid treatment is prescribed as well as identifying new therapeutic pathways to control stress related disorders.

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

The project is multidisciplinary and we collaborate extensively with both basic scientists and clinicians in both the UK and overseas sharing resources, knowledge and expertise. We use multiple platforms to disseminate our work to both the scientific and lay communities ensuring our work is published in open access journals and all data, regardless of outcomes, are made publicly and freely accessible for reuse. This includes

dissemination and sharing of best practice as well as communication of unpublished findings or unsuccessful approaches in appropriate forums including meetings and events that we organise and arrange in the areas of pituitary, endocrine and stress physiology.

Species and numbers of animals expected to be used

- Mice: 6100
- Rats: 650

Predicted harms

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

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

Mice and rats are well validated models for understanding the physiology of the stress axis and retain key structural, signaling and behavioural responses observed in humans, unlike lower order species. The majority of our studies will use mouse as a model to take advantage of the wealth of literature and existing data resources on HPA axis physiology and behaviour. This includes extensive genomic, transcriptomic, proteomic, metabolomic and functional data combined with a wide variety of genetically modified animals required for gene specific targeting, cell identification and characterisation that we have already established. For some assays mice are not optimal (for example for multiple serial blood sampling for hormones such as ACTH that require a relatively large (several 100 microlitres) volume of blood plasma for assay and thus rats will be used in a limited subset of experiments. In both cases male and female animals will be used (as there are sex specific differences in HPA responsiveness) and our focus is on postnatal and predominantly adult animals to identify responses to a variety of physiological and pharmacological challenges. Indeed, HPA axis activity changes with development including a stress hyporesponsive period in early postnatal life thus the majority of our studies are focussed on stable periods during early/mid adulthood in rodents. This timing also is optimal for use of a number of the longitudinal assays such as our ability to monitor real-time corticotroph cell activity in freely moving animals during and after stress.

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

The vast majority of animals (>85%) will be accounted for by generating genetically altered animals and used for isolation of cells/tissues for experiments in a dish. However, to understand the stress response and stress-related disorders requires the complex interaction between the brain, pituitary and adrenal glands. Thus some animals will undergo surgery (for example to implant a device to allow remote monitoring of cell activity or blood pressure), and be exposed to mild stress or changes in diet (e.g having a high fat diet) and taking blood samples to monitor stress hormones. Experimental durations will range from acute (1 h) to several weeks.

What are the expected impacts and/or adverse effects for the animals during your project?
Potential adverse effects include short term pain following surgical procedures which will be significantly reduced with analgesia. Animals exposed to chronic stress, long term steroid treatment or fed a high fat diet show progressive changes in body weight over several weeks that rapidly recovers after the challenge is removed. In addition, some of

the genetically modified animals show mild change such as a staggered walk or reduced bodyweight.

Expected severity categories and the 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 both mice and rats the majority (~ 80%) of animals will be exposed to a mild severity or lower. A small proportion (~20%) of animals will be in the moderate category (eg those receiving surgery or some genetically modified animals).

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

- Killed
- Used in other projects

Replacement

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

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

Understanding the control of stress relies on complex and dynamic interactions between different cells, tissues and organs, as well as with effects of changes to both the internal and external environment such as diet, drugs and ageing. While important aspects of this project will use *in vitro* and *ex vivo* approaches (e.g. pituitary, adrenal & brain slices, primary cultures, clonal cell lines and be informed by computational models) ultimately understanding how the stress axis is regulated and the impact on animal behaviour requires animal use. Importantly, the mechanisms we are investigating are conserved in humans and rodents but not in lower organisms such as worms or flies.

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

Human tissues, transformed cell lines, lower organisms (eg worms/flies) and predictive computational models.

Most experiments (> 80%) will involve tissues and cells collected from animals that will allow us to validate approaches and tools before proceeding to animal work. In addition, we use a variety of approaches to allow full or partial replacement including use of established transformed cell lines, computational tools to both make predictions based on simulations/models as well as bioinformatic approaches to refine our candidate targets. We have thus considered multiple approaches, and will use aspects of them in our studies, including use of established corticotroph cell lines, organ on chip approaches, non vertebrate models as well as predictive computational models of both corticotroph physiology as well as HPA axis function. We will keep updated on replacement approaches through our networks, literature as well as published resources such as the EURL ECVAM search guide and online resources including from NC3Rs and Animal Welfare Centre.

Why were they not suitable?

Each of the above approaches can only be used for limited aspects of our studies and each have their own caveats.

Human pituitary tissue is very difficult to obtain and typically only from patients with severe disease and we cannot manipulate or measure the function of specific cell types in the stress axis of humans

Transformed cell lines such as the ATT20 mouse corticotroph cell line lack specific receptors and signalling pathways critical for normal pituitary function

Key mechanisms we are investigating are human/rodent specific and thus lower organisms such as worms, flies or zebrafish cannot be used

We and our collaborators have developed predictive mathematical models of both pituitary corticotrophs as well as the stress axis. However, while these approaches will allow us to refine working hypotheses, and to make predictions that can be used to guide and optimise the design of animal experiments the models themselves have to be driven by data we generate from studies in animals.

Reduction

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

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

Our estimates are based on a combination of our own usage statistics from previous work, including undertaking pilot studies where new approaches or methods are being developed, as well as use of appropriate publications, guidelines and experience from researchers in the field as well as regulatory, funding and learned societies and agencies (for example NC3Rs Experimental Design Assistant tool, ARRIVE guidelines, Medical Research Council, Physiological Society). The numbers required for upkeep and desired out-breeding of lines are derived from estimates provided by the animal facility. We seek additional advice and updates from biostatistical experts employed at our institution.

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

In the experimental design stage of the project, we perform pilot studies where possible before planning for large cohorts. Tools such as the NC3Rs Experimental Design Assistant (EDA), ARRIVE guidelines and resources from the Medical Research Council are used to inform and optimize reduction in animal use. In addition, insights through personal communication with investigators in the field, publications or veterinary advice, including discussion with the NVS.

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

We have established multiple measures to optimise the number of animals required. Pilot experiments coupled with our previous work and search of literature have determined fundamental differences in male and female responses to stress allowing us to optimize breeding and colony management. Our work informs the development of predictive computational models of both corticotroph and HPA axis function that allows us to define clearer experimental strategies as well as measurable endpoints. Our work is optimised to make multiple measurements from the same animal – for example cell activity, stress hormones and blood pressure or using multiple assays from in isolated cells/tissues tissues (for example molecular, protein and functional assays). In addition, our approaches have optimised the use of genetically engineered animals including validation of cell specific reporters and promoters to define specific cell types and targeted approaches. Our analysis of all datasets is maximized to extract the maximum information possible.

Refinement

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

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

We will use rodents as an animal model to assess stress axis regulation and the outcomes of manipulating stress axis function on behaviour. We aim to understand corticotroph and stress axis function in healthy animals which will provide a foundation for understand stress axis dysfunction in disease and in response to physiological and phamacological challenges such as stress or steroid drugs. Thus, this approach will reduce the number of animals subject to adverse states.

We also utilize wild-type and transgenic rodents without harmful phenotypes as animal models meaning that rodents in the unperturbed state will experience no harmful effects.

The mouse is the species of choice for most of the experiments as our project includes analysis of signaling mechanisms that are human/rodent specific. In addition, a large body of literature and numerous databases characterizing the properties of physiology and the annotated genome already exists allowing us to refine our approaches, methods and models. We and others have optimised approaches to minimise distress, harm or pain to animals used. For example, our chronic stress paradigm is optimised so animals receive a mild stress each day for up to 2 weeks rather than using more stressful stimuli or longer periods of stress. Our surgical approaches are already well validated with appropriate anaesthesia and pre- and post-operative analgesia as well as close monitoring.

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

A key goal of our work is to understand the real time dynamics of corticotroph and HPA axis physiology before, during and after chronic stress and other physiological challenges in adults. Importantly, many of the key signalling components and mechanisms are mammalian specific thus use of lower organism such as flies, worms and fish do not allow

us to interrogate function of biomedical relevance to humans. Moreover, the structure and dynamics of the HPA axis changes during development and thus immature mouse stages cannot be used. Importantly, understanding the real time dynamics of corticotroph and HPA axis function before during and after stress cannot be assessed in terminally anaesthetised animals and anaesthesia itself can modify the stress response. Several of our humane endpoints require animals to both experience and respond appropriately to normal physiological challenges.

Thus, while some key experiments can use terminally anesthetised animals (e.g. monitoring the response of corticotrophs to a bolus injection of a drug) longitudinal monitoring of corticotroph activity during chronic stress and following recovery requires adult sentient animals.

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

Multiple approaches are used to refine procedures to minimize welfare costs.

Studies have been optimised to reduce the duration of exposure to potential harms, for example we use a 2 week exposure to a daily restraint stress minimising both the total duration and types of chronic stressors required.

Regular non-invasive monitoring of animal function such as daily urine to monitor stress hormone levels

Optimised surgical procedures with appropriate pain management and post-operative care (such as using general anaesthesia during, and analgesia following, surgery).

Improved minituarisation of devices and remote recording of multiple physiological functions (e.g simultaneous monitoring of activity, blood pressure and temperature).

Optimised targeted molecular approaches increasing specificity and thus ameliorating potential adverse effects of global genetic manipulations.

Use of ex-vivo tissue/cells for real time physiology (e.g. electrophysiology and imaging) provides clearer mechanistic insight and defined endpoints prior to refinement of approaches to be used to interrogate in vivo.

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

We constantly review the literature (including from systematic reviews) as well as regular consultation of refinement guidance published as part of the ARRIVE guidelines, LASA guidelines, NC3Rs resources and NoreCOPA PREPARE guidelines and recommendations for excellence.

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

We use multiple internal and external resources to stay informed about NC3Rs and ensure effective implementation including: i) Regular discussion with our NVS, NACWO and animal technicians in our dedicated facility. Our institute also coordinate a large number of in-person and digital events and regular communications with updates and best practice.

ii) Regular utilisation of the extensive NC3Rs resources at NC3RS.org.uk including webinars, training programmes, tools and workshops and we subscribe to the NC3Rs e-newsletter for regular updates. iii) we also keep updated of the relevant scientific literature for improvements in our own field (e.g. optimisation of chronic stress paradigms) as well as the broader NC3Rs approaches across new technologies, models and approaches through literature searches and discussions with colleagues, learned societies and relevant funding agencies.

21. Mechanisms regulating energy homeostasis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Obesity, Central nervous system, Body weight, Food intake, Metabolism

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The overall aim of this project is to define the brain circuits that control metabolism through their effects on both feeding behaviour and the organs involved in energy balance. The ultimate goal is to further understand how body weight is regulated and how defects in this regulation may cause obesity and its associated diseases, such as diabetes.

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

Why is it important to undertake this work?

Obesity and its associated conditions, such as type 2 diabetes, are very common causes of disease and death world-wide. Lifestyle interventions such as diet and exercise are often not effective long- term treatments and other therapeutic approaches are needed. However, currently therapeutic options for the treatment of obesity are suboptimal and this in part stems from an incomplete understanding of the mechanisms by which the brain has overall control of metabolism. Given the substantial unmet therapeutic need and the major economic and social burden of obesity and its associated diseases understanding the causes of obesity will help the development of effective treatments and prevention strategies with potentially enormous benefits.

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

This project will provide new scientific information on the brain circuits that are involved in metabolism, food intake and food seeking behaviour. The aim of this project is to provide a deeper understanding of the mechanisms by which the nervous system, hormonal system and metabolic tissues regulate bodyweight, feeding behaviour, and glucose metabolism.

New information will be published in peer-reviewed scientific journals and will inform scientists and clinicians working on understanding the causes of metabolic disease and on the development of improved approaches for the treatment of patients with obesity and its associated diseases. In addition to publication the data will be presented to the scientific community at both national and international meetings.

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

The short term benefits will focus on enhanced understanding of brain circuits involved in keeping energy levels stable, food intake and food seeking behaviour

In the medium term the project aims to identify new potential therapeutic targets that will be useful for future research and for other investigators conducting experiments in a similar area. If we can identify new therapeutic targets in the brain to control feeding behaviour and metabolism then we will use these to try to design further studies to test these targets. This information may also be useful for other scientists involved in the discovery of new drugs and other treatment approaches focussed on the brain. Furthermore, data will be used to design experiments and investigations that will move the field forwards in future work. In the long term, if therapies based on this work can be developed doctors will benefit from the development of improved treatment of patients with obesity and its related diseases. Furthermore, patients may benefit through the development of treatment strategies for obesity which may be drug or behaviourally based.

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

We will collaborate with research groups within our establishment, the UK and worldwide if necessary in order to gain access to the best technologies and expertise. Furthermore, we will share our mouse models and viruses with the wider scientific community.

We will present at both national and international conferences to disseminate our research. We will also publish our work in peer-reviewed scientific journals and will communicate significant findings via the University communications teams to ensure adequate dissemination of our work both to local and international stakeholders. In the past our work has been covered by both the print and online media. Our work has been presented in our institutions annual research report on animal work which is available to the general public.

We will share negative findings by depositing manuscripts on an open access preprint server such as BioRxiv and also publish these finding in regular journals.

Species and numbers of animals expected to be used

- Mice: 39125

Predicted harms

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

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

For the vast majority of studies we will be using adult mice for the experimental parts of our project for several reasons. Firstly they are the lowest type of mammal for which models of feeding, behaviour and metabolism have been well characterised. They also represent good models of human metabolic diseases as the same genetic mechanisms that control metabolism in mice are found in humans. Mice are also prone to develop metabolic diseases such as obesity and they overeat in a similar way to that seen in humans. Mice are also the species that is most amenable to the genetic manipulations that are required for the proposed studies and robust protocols and techniques have been established in mice to study metabolism and metabolic disease. For a small number of studies we will expose pregnant mice to altered diet to assess the effects on their developing offspring. We will study their offspring as adults.

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

The overall aim of this project is to define the brain circuits that control metabolism through their effects on both feeding behaviour and the organs involved in energy balance. To do this we need to use genetically modified mice that allow us to manipulate specific types of neurons in the brain. This may require importation of sperm or embryos from commercial sources or collaborators that will be used to establish these mouse lines in-house using *in vitro* fertilisation (IVF) and embryo transfer into a recipient mother using established standard protocols. Where possible we will use male mice that are genetically altered to make them infertile, rather than having to surgically sterilise male mice. For any surgical procedures appropriate aseptic techniques and analgesia will be used to minimise the risks of infection and pain.

The mice will either be genetically altered by germline (i.e. passed from parent to offspring) or by introduction of a substance (e.g. a virus) which brings about the required genetic changes. The genetic alteration caused by the virus will be specifically in the brain and will be introduced by injecting directly into the brain under general anaesthesia. Our experience with using the genetically modified strains we propose is that the genetic modification *per se* does not cause adverse effects. Appropriate aseptic techniques and analgesia will be used to minimise the risks of infection and pain. Animals will be allowed a minimum period of 7 days to recover from the surgery before they undergo any further procedures. The effect these genetic alterations have on body weight, body composition, food intake, glucose levels, metabolic rate and behaviour will be investigated. These studies may be conducted on regular diet or on a diet where the composition has been altered e.g. a high fat or a high sugar diet, or after administration of substances such as hormones which can alter feeding and metabolic parameters.

An example of a typical study may involve groups of genetically altered animals on regular versus high fat diet with body weight measurements every week, food intake and metabolic rate studies performed at different ages. For food intake studies, it may be necessary to singly-house the animals to determine the exact amount that each animal is eating; animals will be housed like this for the minimum amount of time necessary to gain good quality scientific data. For metabolic rate studies the mice will be placed in a special ‘home’ cage system with water, food and oxygen capacity monitored continually, and assessed for a typical period of 7-10 days.

Alternatively, a study may involve groups of genetically altered animals on regular versus high fat diet with body weight measurements every week and body composition analysis and blood glucose measurements every 8 weeks from 2-6 months of age. For body composition assessment the animals will be placed individually in an imaging machine for approximately 3-5 minutes and then returned to their home cage. For the blood glucose measurements, the animals will typically receive an intraperitoneal bolus of glucose followed by regular collections (over 2 hours) of blood from the tail vein for measurement of blood glucose. Never more than 6 tolerance tests will be conducted in the same animal, with typically a minimum of 7 days between tests. Occasionally we may also need to take blood samples to determine levels of certain hormones (e.g. insulin). We limit the volume taken and limit the number and frequency of these blood draws.

In separate groups of mice, behaviours relevant to energy level balance, such as foraging, appetite and motivation, will be tested in various ways. Some of these tests will require the animals to work for food or liquid rewards. For these tests to work well the animals need to be hungry or thirsty and so we will limit their access to food or fluids prior to testing. Animals will be weighed and monitored daily during this period of limited food or water access and food or water will be supplemented if necessary. No animal will have food and water removed at the same time.

A large proportion of this project is to study brain activity of populations of nerve cells in different brain regions whilst mice are feeding or engaging in a particular behaviour. Therefore, at the same time as administering a virus to genetically alter the animals we will implant a device (e.g. a fiber optic or lens probe) that can detect changes in brain signals. Mice will typically have 4 injections into the brain but may increase to a maximum of 10 depending on the areas of the brain being injected. Mice will typically only have 2 devices implanted into the brain but when necessary this may need to increase to 4. Mice will typically undergo no more than 2 stereotaxic surgery sessions in their lifetime but when necessary this may need to increase to 3. Two to three weeks after the surgery we will connect the mice to fiber optic cables or a miniature microscope so that we can visualise brain activity while they engage in a particular task. In some cases we will implant a small plate to the skull of the animal so that we can temporarily restrain them by the head while they are free to voluntarily move on a platform suspended on an air cushion. Mice adapt very well to this, and it enables us to conduct specific behavioural tests e.g. responding to a sensory stimuli such as a smell.

In other experiments, we will shine laser light through the implanted fibre optics when the animals are engaging in a particular task. This light activates special proteins that are expressed by the injected virus and cause the nerve cells to become activated. This will allow us to determine if these nerve cells are involved in a particular behaviour e.g. feeding or foraging.

In some studies we will give hormones or other substances to mice including psychoactive drugs and monitor their effects upon feeding and related behaviours as we are interested in the links between food intake and emotional behaviours. In some cases these may need to administered chronically and in order to avoid giving mice multiple injections these substances may be administered via slow- release pellets or subcutaneous osmotic mini-pumps.
For aversive learning we may administer footshocks. The shock levels we use are as low as is practicable and cause only brief peripheral discomfort (an unpleasant prickling sensation in humans) rather than serious pain. We will typically train mice with 1-3 shocks, but in some cases may need to increase the number to 5.

Typically testing will take no longer than 6-9 months and after completion animals will be humanely killed and their organs taken for analysis.

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

All expected impacts or adverse effects will be monitored carefully by a licensed member of our team alongside a named veterinary surgeon and named animal care and welfare officer.

The majority of animals are expected to experience no negative impact or adverse effects after procedures. We do not anticipate the genetically modified mice to have harmful phenotypes as we have used most of these strains before. All animals that have surgery are expected to make a rapid recovery within a couple of hours, and any sign of pain or distress will be rare. We do not expect to observe any neurological changes in the animals after administration of substances into the brain or after implantation of devices, such as fiber optics, into relevant brain areas. For some studies we need to treat mice with drugs that induce the expression of genes. One of these is a hormone that may cause irritation at injection sites or gastrointestinal disturbances, but these are rare and we will carefully monitor for them and intervene as required.

Animals that are fed a high fat diet may develop a greasy coat that tends not to bother them, although in rare cases they may develop slight skin damage.

Animals may suffer from mild and temporary discomfort at injection sites if substances are administered. If injections are repeated this risks pain, distress and increased sensitivity. To minimise these we limit number and frequency and vary injection site. Use of minipumps very occasionally leads to inflammation around the pump. We will monitor carefully and intervene as required.

Administration of endogenous hormones or peptides or substances that act at endogenous receptors will cause transient alterations in food intake, which will not significantly affect the body weight of the animals. However, if administered chronically we may see increases and decreases in body weight dependent on the substance being administered but we do not expect this to be to the degree that would affect their welfare. Animals will be monitored and weighed regularly.

The psychoactive drugs we plan to use will only cause a transient increase in activity, similar to levels that are seen in mice at night. Activity levels should return to normal levels within a short period of time (2-3 hours). If these drugs are given chronically then it will usually be for a maximum of 7 days as we have done previously. This has not elicited any adverse effects other than hyperactivity in the past. We have also not observed any signs of withdrawal with the doses used in any animals.

Animals that undergo enforced fluid or food restriction may experience weight loss or dehydration. Any animal who drops below 85% of their lean starting body weight for a period of 24 hours will be given additional food or water.

All tests for determining alterations in metabolism, imaging and behaviour are not expected to have any negative impact, and any sign of pain or distress will be rare. For some studies we will alter the activity of neuronal pathways that control anxiety as they overlap with the pathways that control feeding but our experience is that such behaviour is

transient. We do not anticipate seeing seizures in such studies as we activate discrete populations of nerves: to date we have not seen such effects in our studies on similar brain regions. In some studies we will reduce the caging temperature that mice are exposed to but we will acclimatise the mice to this and carefully monitor bodyweight, food intake and behaviour during these studies.

In most cases animals will be group-housed for experiments. However, in some cases (e.g. when determining food intake) it may be necessary to singly-house them. If this occurs then they will be singly-housed for the minimum time possible and will be given environmental enrichment in their cages. Their weight and general behaviour will be monitored.

After administration of insulin, animals will be closely monitored to make sure that they do not suffer a hypoglycaemic event (when blood sugar level falls too low for bodily functions to continue).

Footshocks are a well-established method to induce aversive learning with very limited exposure to the aversive stimulus, and therefore makes it a preferred method for studying aversive learning. The shock levels we use are as low as is practicable and cause only brief peripheral discomfort (an unpleasant prickling sensation in humans) rather than serious 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)?

All mice used within this license are expected to fall under the sub threshold, mild or moderate severity categories. Based on our experience and the experiments planned we expect these to be divided as follows:

Sub threshold: 45%

Mild: 15%

Moderate: 40%

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

- Killed

Replacement

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

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

The regulation of feeding, behaviour and metabolism in mammals is achieved by a complex system that includes the nervous system, key tissues that regulate metabolism such as the liver and body fat. In addition, a wide range of hormones, substances involved

in metabolism, and other substances that circulate in the blood stream, act inside the brain and allow this system to be responsive to changes in the environment. Furthermore, the end outputs of these systems, such as eating a meal or behaving in a particular way or changes in bodyweight and metabolism, are features of intact living mammals.

Therefore, the study of such complex systems, and how they work normally and go wrong in disease, requires the use of whole animals in which all these systems are still in place and talking to each other. While some features of these systems can be studied using animal tissues in a test tube breaking such things as the connections between the brain and tissues means that such approaches only give a limited insight into how these systems work. Finally, as we wish to eventually extend such studies to include humans and patients who are necessarily studied as a whole organism then studying intact mice is the best approach to build an understanding of human functions and disease.

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

We considered using cell models, flies and computational approaches.

Why were they not suitable?

Long-term cultures of cells in the laboratory do not demonstrate the key features that are involved in metabolic regulation. This is particularly the case for nerve cells. In particular, the cells are not exposed to the hormones and nutrients and other features of the metabolic environment that are found in an intact animal. Furthermore, nerve cells in an intact animal are organised into complex circuits but this is not possible to achieve in a cell culture dish. Finally the features that we are interested in studying such as feeding and behaviour are not possible to study in simple cell preparations. Some behavioural processes can be studied in intact fly models but such systems do not reflect the complexity of mammalian biology and in particular the brain functions involved in many aspects of feeding behaviour. Modelling metabolism and complex brain function and behaviour has to date not been possible using computer based approaches.

Reduction

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

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

We have used the NC3Rs Experimental Design Assistant and statistical tools to carefully design our experiments and determine the minimum number of animals that we will need to achieve the scientific aims and objectives of this project. These numbers are further supported by our extensive experience with studies conducted under our previous Home Office Project Licence that used similar experimental approaches and mouse models

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

In addition to being able to draw on our significant prior experience and published work, we have consulted the NC3Rs experimental design assistant allowing us to calculate the appropriate sample size based on each experiment. This will ensure that we are using the fewest number of animals and that each animal is being utilised to its full potential. We frequently design our studies so the same animals are used in both control and experimental studies (crossover design) and this reduces the numbers used. We use virus-based techniques to alter gene function when possible so that we can reduce breeding of mice with genetic manipulations. When planning studies we make use of published databases that describe the genes in cells and the connections between neurons so we can focus our studies on particular cell types and brain regions reducing the number of mice used for exploratory studies. We use databases of existing mouse resources and obtain mice from these archives as embryos or sperm rather than generating mice ourselves.

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 monitor our breeding colony to ensure that we are correlating our sample sizes for experiments with the number of animals being produced, to avoid breeding unnecessary animals. We undertake pilot studies with a limited number of mice to establish the anatomy and existence of connections between specific nerves and the patterns of genes expressed in cells using brain slices before starting upon studies of feeding and behaviour in whole animals. Such pilot studies allow us to focus upon particular cell types and brain regions.

Refinement

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

Which animal models and methods 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 in our studies. These will include both wild-type (i.e. 'normal') and genetically altered mice with the latter largely obtained from existing commercial and academic sources. We will study mice with loss and gain of gene function (mainly in specific cell types) and also mice which express genes that allow specific cell types to be 'labelled' so that we can either manipulate them using viruses or identify them in studies of anatomy. By mainly targeting specific cell types we minimise the chance that genetic manipulations themselves have broad effects on animal well-being. With the models we use there is no pain, suffering, distress and lasting harm from the genetic manipulations themselves.

The methods we will use will include surgical approaches to allow us to inject viruses into mice. Such surgery is performed by highly experienced people using the best techniques under sterile conditions and general anaesthesia. We closely monitor animals during surgery keeping them warm and hydrated and after surgery give mice post-operative pain medication, antibiotic and hydration as required. We also give mice injections and these will use the best route and the lowest volume required. When taking blood from mice we

will again take the minimum volumes required using established best practice. For studies on feeding and behaviour we employ well validated approaches and acclimatise the mice to the studies and apparatus so that any distress to the animal is minimised. Many of our approaches use apparatus that permits remote monitoring of behaviour so that mice can go through the study without being disturbed by the investigator. It is important in studies on feeding and behaviour that the mice are familiarised to the experimenter and the apparatus as feeding and behaviour are sensitive to stress. Therefore to make sure our studies are robust we employ the best approaches to such studies. We provide mice environmental enrichment to make their cages more interesting and allow mice to live in groups to again maintain a good environment for them.

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

We wish to study feeding and related behaviours and metabolic regulation in adult mice with a view to understanding the potential causes of obesity and diabetes in humans. We therefore need to study free-living animals with the same level of brain complexity and metabolic regulation seen in mammals and humans. Thus we cannot use a more immature life-stage or species that are less sentient. We also cannot study feeding and behaviour in mice that have been terminally anaesthetised as such animals are not capable of undertaking these activities. Furthermore anaesthesia has effects on metabolism that would interfere with getting results that are relevant to normal function in mammals.

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

We closely monitor animals during surgery keeping them warm and hydrated and post-operatively proactively give mice post-operative pain medication, antibiotic and hydration as required. Where possible we will give substances to mice by mouth rather than giving multiple injections. For studies on feeding and behaviour we employ well validated approaches and acclimatise the mice to the studies and apparatus so that any distress to the animal is minimised. We make extensive use of this training of mice to make sure they are not distressed during the studies. We undertake long term studies such as imaging of brain activity which benefit from such acclimatisation protocols, provide high quality data as well as reducing animal usage. We also make increasing use of approaches that employ apparatus that permits remote monitoring of behaviour and other areas of interest so that mice can go through the study without being disturbed by the investigator.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
We follow governmentally published documents such as the Guidance of the Operation of the Animals (Scientific Procedures) Act, the ARRIVE guidelines, LASA guidelines (e.g. on administration of substances, blood sampling and aseptic techniques), the relevant published literature on studying metabolism in mice as well as the PREPARE guidelines, NC3Rs website and published guidelines from the local 3Rs advisory group.

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

We will consult with such resources as the NC3R database and the local 3Rs advisory group. We will consult regularly with colleagues, CBS staff and vets about best practice and potential further refinement of our procedures. There are regular updates to how to

study mouse metabolism in the published literature and we will follow the published best practice.

22. Glia contribution in neurodevelopmental disorders

Project duration

5 years 0 months

Project purpose

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

Key words

Brain, Neurodevelopmental disorders, Myelin, Neuron, Glia

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to investigate the interactions of neurons with the surrounding brain cells (called glia cells) during normal brain development and when the development of the brain is disrupted leading to disorders called neurodevelopmental 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?

In the UK approximately 45 million cases were characterised as "brain disorders" with an estimated cost burden of £100 billion, the largest of which in terms of cost belongs to neurodegenerative, neurodevelopmental and neuropsychiatric disorders. Developmental

disorders of the brain (neurodevelopmental disorders) are often accompanied by autism and intellectual disability. These manifestations can be severely limiting, and they emerge after a disruption in the communication between neurons (nerves) or between neurons and surrounding cells (glia) in the brain. Autism Spectrum disorders is a group of neurodevelopmental disorders that develop in one in 100 children, affecting approximately 700.000 people in the UK. Autism spectrum disorder symptoms cover a wide range including stereotypic behaviours, poor cognitive functions, social and communicational deficits that develop early in life and persist until adulthood. These symptoms can be severely limiting for affected individuals, but we currently lack effective treatments.

The major benefit of this research is the identification of new drug treatments for people with autism- associated neurodevelopmental disorders. My research focus on glia cells called oligodendrocytes and their communication with the neurons they contact takes a new direction on neurodevelopmental disorder research that is now imminent if we wish to understand them. Oligodendrocytes produce myelin, a substance that covers the neurons and has a similar role to the insulation of wires, protecting the neuron. So far, how this insulation is affected and what happens to the underlying neuron in neurodevelopmental disorders has not been addressed. However, evidence from brain scans in humans and rodent models point to abnormalities in this insulation.

The goals of this project are:

To identify the specific molecular and cellular pathways that are affected in oligodendrocytes using rodent models of neurodevelopmental disorders.

To understand how these changes in oligodendrocytes affect the communication with neurons and how they lead to the behaviours that we see in neurodevelopmental disorders.

To target and restore the affected pathways with the idea to develop therapies that will restore the normal brain function.

This project will recruit a group of scientists and will generate new knowledge with the hope that it will be then translated into new strategies for the design of better treatments for people with neurodevelopmental disorders. As new therapies develop within the years the burden on affected people and their families will be progressively lifted and the quality of life will be significantly ameliorated.

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

This project will determine:

The mechanisms by which gene mutations causing neurodevelopmental disorders affect oligodendrocyte function and myelin formation during brain development and in the adult.

The effect mutant oligodendrocytes have on the insulation, nutrient supply and function of neurons.

The contribution of mutant oligodendrocytes to the apperence of behaviours associated with neurodevelopmental disorders.

The type and timing of effective oligodendrocyte manipulations for the restoration of normal brain function.

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

This project takes a new direction in the field expanding the current knowledge from neurons to neuron- glia communication in the context of neurodevelopmental disorders. Understanding how glia cells such as oligodendrocytes contribute to the formation of healthy brain networks during development will unfold new possibilities for effective treatments for neurodevelopmental disorders using a different route: that of glial cell manipulation. In addition, the project will assess the likelihood of currently available therapeutics to be re-purposed to treat neurodevelopmental disorders associated with autism and this would be the major benefit of this project. The identification of novel treatments, or the enhancement of existing therapies, would have a significant positive impact on affected individuals and their families by increasing their quality of life, while reducing the cost of care for affected individuals.

Another benefit of this project is the advancement of scientific knowledge on the mechanisms of neuron-oligodendrocyte communication. The beneficiaries of this include other scientists in field, as well as members of the institution and UK research councils who will benefit from any high-impact papers based on this research.

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

To maximise the outputs of this work I will disseminate the findings of this project with:

High-Impact peer-reviewed publications

Presentations in national and international scientific conferences

Collaborations with basic and clinical scientists of the scientific community

Public engagement events

Species and numbers of animals expected to be used

- Mice: 7500
- Rats: 4500

Predicted harms

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

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

This project aims to better understand how neurodevelopmental disorders are caused and how we can reverse them. In humans, autism-associated neurodevelopmental disorder symptoms become evident in early post-natal life and persist throughout adulthood. It is therefore critical to study these disorders both during development and in the adult to understand not only how but when we can intervene.
Neurodevelopmental disorder biology is quite complex since different cell types in the brain carry the responsible genetic mutation. Each mutation can affect differently the development of neurons and that of glia cells and completely alter the ways these cell populations interact and form bigger brain networks. Mice and rats share more than 97%

genetic similarity with humans and the genetic mutations that cause neurodevelopmental disorders in humans affect the rodent brain in a similar way that we can reproducibly study. In addition to the genetic similarity with humans, rodents also have similar brain development with that of humans and show behaviours relevant to human neurodevelopmental disorder-related behaviours that we can monitor. For these reasons, the rodent models of neurodevelopmental disorders are ideal for us to study how these disorders develop before the appearance of symptoms and later on, for the identification of the optimal time-points for therapies.

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

We aim to study the interactions of oligodendrocytes and neurons in the healthy brain throughout brain development until adulthood and compare them with those in the brains that develop a neurodevelopmental disorder. For this reason, experiments will include the administration of substances or viruses that express fluorescent proteins to pregnant dams, neonates, juveniles, and adults or the use of genetically altered animals that will help us visualise and follow these cell types during brain development.

On some occasions we will need to manipulate either the number, the function of brain cells or the signals they receive from other cell types and the environment. This will help us to discover the signalling pathways and the cell functions that are important for a healthy brain network and how and when these change during neurodevelopmental disorders. This will involve the administration of drugs, substances in different stages during brain development, the administration of viruses that affect the expression of various genes and signalling molecules and the use of genetically altered animals with altered gene expression. Animals will receive drugs and substances via the oral route or via the intraperitoneal, intravenous, intramuscular, intranasal or subcutaneous routes. When this is not possible a surgery will be necessary to deliver them directly in the brain. The administration of viruses that change the expression of molecules will be done intravenously or by surgically injecting them in the brain. Animals will be then studied for a period of weeks or several months after each intervention.

To test if the transplantation of glial cells is beneficial for the restoration of healthy brain networks in neurodevelopmental disorders, some neonatal animals will get injections of cells in specific brain areas that will be monitored for a period of several weeks or months.

To test if each of the above interventions positively affects animal behaviour and therefore prevents the appearance of neurodevelopmental disorder-related symptoms, we will need to perform behavioural tests in the animals several days, weeks of months after the intervention. On rare occasions animals will need to have two separate surgeries (for example one to receive cell transplantation or a virus that labels cells followed by the administration of a drug or a substance later) separated by a period of time that will allow animals to recover before the second surgery is performed. None of the experiments will be performed in aged animals. Several experiments may end with animals being killed under terminal anaesthesia to allow fixation and removal of tissues for further examination or culturing of the different cell types in a dish.

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

We will breed animals with disruptions in genes linked to neurodevelopmental disorders. We expect few animals to suffer from the effects of these genetic alterations but we will conduct most of our experiments before these adverse effects appear. The animals will be

killed by humane methods and their tissues used after their death to address the experimental questions of interest. We will also use animal tissue to obtain cells and sections of brain to grow in a dish to test possible therapies. This will also involve surgery for the administration of compounds in the brain when another route is impossible, which is of moderate suffering. To minimise this suffering these procedures will be carried by trained personnel using pain relief under NVS advice, and the animals will be closely monitored for signs of harm. In some cases, animals may be given drugs to study the effects on molecular, cellular, electrophysiological (properties of signal transmission in the brain) and behavioural processes, but it is not expected that this will cause any adverse effects other than mild clinical signs (i.e. decreased appetite, lower levels of activity). In some cases, we will test animals for their performance on learning and memory tasks. This will be necessary to reveal potential benefits from our drug treatments and animals should not experience any adverse effects other than mild clinical signs. All animals will be humanely killed at the end of the experiments.

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

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

Of the genetically altered mice and rats bred and maintained on this licence some 70% should experience MILD severity or lower, with 30% experiencing MODERATE severity. All the rats and mice killed to obtain nervous tissue for other experiments will experience severity no greater than MILD. Of the mice and rats that undergo behavioural evaluation of the effects of the various genetic or pharmacological manipulations during brain development, some 60% are likely to experience MODERATE severity, with the other 40% not exceeding MILD severity.

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

- Killed
- Used in other projects

Replacement

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

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

The aims of this project require the molecular/cellular and electrophysiological study of the mammalian brain and the assessment of complex behaviours to better understand how neurodevelopmental disorders are caused and how we can reverse them. Complementary work using culture systems from human derived brain cells will be performed when possible and will be a powerful tool especially for addressing simple biological questions and the effectiveness of potential treatments. This technology is new and a great replacement for animal work but still unable to faithfully replicate the complexity that we find in an intact mammalian brain. Therefore, we still need to study the intact mammalian brain using animals. Mice and rats share more than 97% genetic identity with humans and the genetic mutations that cause neurodevelopmental disorders in humans affect the rodent brain in an analogous manner that we can reproducibly study. Nevertheless, we will

always use human derived culture systems, human post-mortem tissue whenever available and pre-existing -omics databases for the investigation of molecular pathway changes prior to performing experiments using animals.

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

Non-animal alternatives:

Culture and co-culture systems using human-derived cells

Available databases for the investigation of molecular changes in neurodevelopmental disorders

Human post-mortem tissue

Why were they not suitable?

Datasets are very useful to find key molecules and molecular pathways that can be important for neurodevelopmental disorders.

Culture and co-culture systems using human-derived brain cells are also useful to address simple biological questions and pharmaceutical compounds before any experiment in animals. However, the brain is a three-dimensional structure with many interacting cells and complex networks that we cannot yet model in a dish or maintain for long enough periods to study neurodevelopmental disorders. For these reasons we still need to turn to animals, and in particular, mammals, to help with this complexity.

Human post-mortem tissue is restricted by the limited availability of tissue of adequate quality.

Reduction

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

For this project we will use minimal animal numbers to produce robust and reproducible scientific outcomes, considering severity. The numbers of animals that will be used for this project were estimated after statistical analysis calculations of previous data using specialised computer software.

This project requires the maintenance of a significant number of animals because:

According to our calculations, group sizes or at least 15-20 animals are required for most of the proposed experiments.

Certain experiments will require the use of at least 4 separate groups (for example when a specific drug is compared to placebo in GA animals and controls)

In some of the GA animal groups that will be analysed, the gene of interest is carried on the X chromosome and therefore it would not be possible to equally use animals from both sexes.

For the proposed behavioural and electrophysiological analyses, each will require a different cohort of animals.

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

Experiments will be performed according to the ARRIVE guidelines 2.0. In order to reduce the animals being used in this project, the following were taken into consideration:

Formulation of clear experimental questions, assignment of suitable controls, and independent replications for each experimental question.

Efficient experimental design (such as factorial, randomised block, unequal groups) was considered to reduce suffering and animal numbers.

Power analysis calculations of previous or small group data were and will be used prior to the assignment of larger animal groups for adequate power to detect genuine scientific differences.

To avoid bias groups will be assigned in a randomised fashion and experiments will be performed blind to genotype and treatment using codes kept by a person not involved in the experiment and file randomiser software such as the Fiji software File randomiser macro.

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

To further optimise the number of animals for this project we will:

Ensure that trained personnel perform the experiments in communication with the animal units to ensure good health, minimum animal stress and less variability.

For many experiments cultures of brain cells and brain slices will be used prior to any experiment in animals.

For the experiments that involve brain tissue, cell cultures and slices, the same animal material can be used for multiple experiments and by multiple 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.

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

Mice and rats have more than 97% genetic similarity with humans and the genetic mutations that cause neurodevelopmental disorders affect the mouse and rat brain in a similar manner that we can reproducibly study. Both mice and rats are the most tractable models for the in vivo study of neurodevelopmental disorders to determine the molecular, functional, and structural characteristics of brain networks. Mice and rats develop a range of neurodevelopmental disorder-like behaviours that are relevant to the respective human ones. However, the two species display distinct phenotypic characteristics for each neurodevelopmental disorder at the cellular and functional level and therefore it is critical to include both if we aim to find effective and relevant treatments for the human neurodevelopmental disorders.

For instance, the genetically altered model rats that have been generated for several forms of neurodevelopmental disorders develop more complicated cognitive disruptions that cannot be observed in mouse models. This is because rats have better developed brain areas than mice, responsible for these behaviours. At the same time, there are more available mouse genetic tools and commercially available reagents we can use to study the molecular mechanisms that underlie neurodevelopmental disorders which are not available for rats.

Hence, mouse and rat models of neurodevelopmental disorders have been established and show high relevance to the human neurodevelopmental disorders, some of which will be used for this project. The use of mice and rat models of neurodevelopmental disorders is a refinement to the use of other higher mammalian organisms as they have a lower degree of neurophysiological sensitivity and exhibit less severe behavioural symptoms.

Nevertheless, some of these genetically altered animals do develop moderate symptoms. As a refinement we will use established severity scales and monitoring systems that have been tailored specifically for each line. Each of them contains distinct trigger points (i.e. for instance ≥10% of weight loss over a period of 1 week) that will dictate necessary interventions for the improvement of animal welfare (body weight monitoring twice a week and administration of food supplement), clear observational points that define the severity (i.e. phenotypic scoring scales including mobility, gait, tremor, breathing and general condition) for each line, and well-described humane endpoints (such as ≥20% weight loss or a total score of 15 in the severity scale) that will lead to the humane killing of the animal and the termination of the procedure.
The animals in these studies will be cared by trained staff within a well-resourced and well-equipped modern animal facility with adequate environmental enrichment. Half of the methods that will be performed for this project include the in vitro culture of neurons and oligodendrocytes or the culture of brain slices. These methods are also used as a refinement for the use of in vivo models since they will allow us to study the behaviour of normal and genetically altered cells and the molecular mechanisms that underlie them using tissue derived from humanely killed mouse and rat pups and therefore minimising the pain and harm to the animals. The same systems will be used for testing the efficiency of viruses, drugs and cells before any administration in vivo. Only the more effective drugs/viruses/cells will be administered in vivo. As an additional refinement, we have chosen to test the efficiency of drugs and substances that have already been successful in other pre-clinical models and/or already used in human clinical trials. Tissues that will be used for the histological and electrophysiological analysis will be obtained by animals that will be humanely killed.

In some cases, we will need to use surgery to deliver drugs/viruses/cells directly in the brain when another root of administration is not possible. For each surgery we will use aseptic conditions to minimise unnecessary and harmful inflammation and post-operative analgesia to reduce pain and suffering after the surgery. Animals will be closely monitored and scored by well-trained staff and will be allowed to fully recover before any subsequent procedure. These short-term surgeries are usually well tolerated by the animals and induce only transient suffering that we expect to minimise with the post- operative care and analgesia. Finally for the assessment of the animal's behaviour we will perform behavioural tests that are not expected to cause any adverse effects other than mild or transient discomfort. Any animal that will show any unexpected signs of suffering that is not minimal, or transient will be humanely killed.

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

A major goal of this research is to identify and correct the disruptions in neuron-glia communication in neurodevelopmental disorders using pharmacological and genetic strategies. The use of the genetically altered rodent models is critical to this goal because they effectively model the human mutations that cause these disorders and because it is necessary to study the complex interactions of an intact mammalian brain.

Even though the oligodendrocyte generation and migration to different locations in the brain happens early in development, the insulation of neurons with myelin is primarily a post-natal process that continues into adulthood in vertebrates. It is therefore critical to follow this process in time something that is not possible using less sentient and less evolved animals. In addition, complicated cognitive functions and behaviours are closely linked to neurodevelopmental disorder research. These behaviours and the effectiveness of treatments cannot be assessed if animals under terminal anaesthesia. Finally, this project requires the use of genetically altered rat models that will allow us to assess more complicated cognitive disruptions that cannot be observed in mouse models, due to the better developed brain area responsible for these behaviours in the rat, the prefrontal cortex.

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

The animals in these studies will be cared by trained staff within a well-resourced and well-equipped modern animal facility with adequate environmental enrichment. For the animal lines with expected moderate phenotypes we will use established severity scales and monitoring systems that have been tailored specifically for each line. Each of them contains distinct trigger points (i.e. for instance ≥10% of weight loss over a period of 1 week) that will dictate necessary interventions for the improvement of animal welfare (body weight monitoring twice a week and administration of food supplement), clear observational points that define the severity (i.e. phenotypic scoring scales including mobility, gait, tremor, breathing and general condition) for line, and well-described humane endpoints (such as ≥20% weight loss or a total score of 15 in the severity scale) that will lead to the humane killing of the animal and the termination of the procedure. Onsite veterinary assistance will provide advice if and when necessary.

Tissue for the in vitro, ex vivo, histological and electrophysiological assessment of cellular functions will be obtained from humanely killed animals. In some cases, we will need to use surgery to deliver drugs/viruses/cells directly in the brain when another root of administration is not possible. For each surgery we will use aseptic conditions to minimise

unnecessary and harmful inflammation and post- operative analgesia to reduce pain and suffering after the surgery. Animals will be closely monitored and scored by well-trained staff and will be allowed to fully recover before any subsequent procedure. Onsite veterinary assistance will provide advice if and when necessary.

Measures of distress for all animals will be assessed (such as hunched posture, abnormal feeding and drinking, weight loss, levels of animal activity). All animals will be carefully monitored and if there is any evidence of suffering greater than minor and transient or in any way compromises normal behaviour, they will be killed using humane methods before reaching the humane endpoints (the humane endpoints are well defined for the models that we plan to use).

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

- Published best practice guidance will be obtained by: Local AWERB guidelines
- ARRIVE and PREPARE guidelines of the NC3Rs
- Guidance on Animal Testing and Research from the Home Office
- LASA and RSPCA guidelines

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

We will stay informed about the advances in the 3Rs by:

- Communication and frequent discussion with the NVS, NACWO and NIO. Attending on-site or online NC3R seminars.
- Registered for NC3R newsletters. Frequently visiting FRAME, LASA websites.
- Attendance of scientific meetings and continued review on scientific literature for the identification of new emerging technologies that can minimise suffering and replace animal use.
- Updates on animal handling and enrichment through http://www.procedureswithcare.org.uk and www.enrichmentrecord.com

23. Provision of an outsourced drug discovery platform for the development of therapeutic drugs for 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

neurodegeneration, stroke, brain, neuron

Animal types	Life stages
Rats	adult
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to test the efficacy of drugs (how well they produce a desired effect) designed for use against human diseases of the brain, such as Alzheimer's and stroke.

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

Why is it important to undertake this work?

Neurological disorders involve damage to the brain, spinal cord or nerves; they can be progressive or sudden, intermittent or unpredictable. They can cause significant burden to the patient, their families, and society as a whole; 14% of the social care budget in England is spent on people living with neurological conditions, so it is a group of conditions that need significant investment and patient care.

Conditions involving neurodegeneration (when brain cells are progressively or immediately damaged or lost) are particularly devastating for older people. The leading cause of death in the UK in 2018 was dementia and Alzheimer's disease, accounting for 12.7% of all registered deaths (Office for National Statistics; ONS). This is expected to rise year-on-year as the population grows and lives longer. Patient quality of life is usually affected more as the degeneration progresses. Life expectancy changes with age of diagnoses, but on average 4.5 years is the median (average) for survival from onset of dementia.

Ischemic stroke is where a blockage (a blood clot) causes a sudden loss of blood supply to an area of the brain, and accounts for 85% of strokes. A stroke can cause various changes to movement, speech, or cognition (the way your brain understands, organises and stores information). The number of deaths through ischemic stroke have almost halved in the last twenty years, most likely through greater wider public knowledge of spotting early signs and therefore providing early intervention. Despite this, the incidences of stroke are set to rise due to an aging population; age-related diseases, such as heart disease or diabetes can increase the risk of having a stroke. Furthermore, quality of life for stroke patients is significantly affected and, in a large proportion, permanently life-changing with the significant risk of a second stroke also a factor in the continued management of the disease in these patients.

Our work aims to use animals, in combination with experiments that take place in the laboratory, to support the development of new treatments for neurological disorders and address the unmet clinical needs for treatments. The use of animals is currently an essential part in the process of developing new treatments and enables us to replicate complex aspects of the disease in the entire biological system. For neurological disorders this includes the interaction of brain cells directly affected by damage, with other nearby cells, and the effect on the animal as a whole (for example how its movements are changed by the disease). They enable us to test the effect of new treatments in a relevant and intact biological system. This work will enable key decisions to be made regarding which treatments will continue to be developed, and ultimately, which will make it into clinical trials, where they are tested in humans. Our approach to the work ensures that this is done in the most efficient way possible, and that the benefit gained from every animal is maximised.

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

Work carried out under this licence will involve the testing of new drugs to treat neurological disorders (diseases of the brain) in a way that is closely related to human disease. As part of this project we expect to test the effects of at least 5 new drugs for neurodegeneration (diseases like Alzheimer's), and at least 3 new drugs for treating stroke. Our clients are experts in developing their potential (candidate) drugs, and our bespoke studies that closely replicate both disease and potential ways of treating the disease. Combining these two approaches, the candidate drugs have significant potential to enter clinical trials. The way the drug works (mechanism of action), or the kinds of biological processes we look at (targets) will be different depending on the client we have, but the aim of treating the specific diseases will remain the same.

This project will provide important information to progress new treatments through the phases of drug development. New test agents will be evaluated for their ability to treat animals with symptoms that closely match those in human neurological disorders. The information gathered will enable us to identify the most appropriate treatments to take forward to human clinical trials. The information gathered will also enable us to quickly determine which drugs should not be progressed any further.

In addition, this work will increase our knowledge of how new drugs work and will help us to identify changes in the body that occur in response to the drug. We can use this information further down the line to monitor responses in humans during clinical trials.

Data from studies under this project may be used to support patent applications and applications by clients for additional funding. Data produced may also support the design of regulatory studies for clients.

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

This project will generate important data in the development of new drugs. To assist with this, we will utilise our test agent dosing project licence to allow us to understand how drugs distribute in the body after dosing, how quickly they are eliminated, and the dosages that are well tolerated in rodents. This will aid in the design of dosing strategies for studies under this licence. Ultimately, work carried out under this licence is expected to result in the progression of new neurological disorder treatments through various stages of drug development and ultimately into the clinic to treat patients.

Our focus on a science-led approach will enable key decisions to be made at each development stage on whether a test agent is likely to become a successful drug. This allows unsuitable drugs to be abandoned at an early stage and enables us to use the fewest number of animals possible per drug development programme. The identification of test agents as unsuitable for use in humans at an early stage of development will ensure a better success rate in the drug discovery process than has been seen previously in the pharmaceutical industry. Ultimately, this project will contribute to the successful development of new drugs, which will benefit patients with various neurological disorders who may otherwise have their quality of life greatly reduced by their condition.

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

All studies are designed such that the outputs from each animal are maximised. Expert knowledge is gathered not only from within the preclinical (animal) team performing the animal studies, but from other teams within our company, or our clients' companies. This ensures that all relevant work that has been performed in the laboratory is taken into consideration when designing animal studies. The in vitro (in the test tube) and bioanalysis teams at our company are experts at analysing tissue and blood samples collected from animals, and they help with details of sample collection and storage to ensure that the samples are collected and stored in the best way possible. They are also experts at working with small quantities of samples, particularly small volumes of blood samples, meaning that they can often analyse lots of different biomarkers (a measurable indicator of a disease state or other physiological state) and test agent levels from each animal.

In addition, we will seek expertise from our established networks, to ensure that we make use of any new knowledge or incorporate better methods of performing animal studies. We will also use these networks to provide information and training to others on the models and techniques we use in our research. We will maintain good communication with

managers of the animal facilities to ensure that any tissues from animals being killed that are not required for our work can be made available to other researchers if suitable.

Although there are times where we will not be able to share animal model information (for example, where it would put us at a competitive disadvantage), we aim to publish or share our findings (especially control data) wherever possible. Likewise, we hope to pass on any 3Rs progress we make in real time to colleagues, but this will ultimately be available locally to other researchers through the AWERB retrospective review for this project.

Species and numbers of animals expected to be used

- Mice: 300
- Rats: 800

Predicted harms

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

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

Mice and rats are the most common type of animal used for developing new treatments for neurological diseases. These rodents are well characterised, meaning that a lot is already known about how their bodies work, and the techniques used to mimic the human diseases are well developed. This means that for the brain diseases we wish to study, using rats and mice will produce data that is very close to how the diseases behave in humans.

Adult rats and mice will be used for the work outlined in this project licence as we want the biology of the animals to be fully developed as this will better represent the patients we aim to treat. Both rats and mice are needed are needed for this project; each species has different characteristics that suit different types of experiments (for example rats are better suited to studying behaviour). Also there are sometimes differences in how each species reacts to the substances used to bring about the diseases (for example one drug we use is tolerated by mice but is toxic to rats).

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

The majority of animals will be part of studies that aim to test whether new drugs can be used to prevent or halt neurological damage associated with stroke or neurodegeneration. Therefore, each animal will be given some kind of new drug at some point during a study, either once or several times. Drugs will be administered more commonly intraperitoneally (into the body cavity), subcutaneously (under the skin) or orally (by mouth), and less frequently intravenously (into a vein), via a slow-release device under the skin, or very rarely into the nose. For intraperitoneal, subcutaneous and oral administration, mice will be held securely by a trained researcher for the dose to be administered. For intravenous dosing, mice will be placed briefly in a specially designed rodent restrainer (a ventilated Perspex tube where the animal can be observed), the tail will be warmed using warm water or more commonly by being placed into a warming cabinet to dilate the blood vessels and make them easier to see before administering the dose. For intranasal dosing, mice will be briefly anaesthetised and placed onto their backs to allow the dose to be administered directly into the nostrils.

The dosing of new drugs should not cause significant harm or distress overall; dosing might be once a day for a week, or once a week and cause only brief discomfort each time.

All animals will either have surgery on one occasion or be given certain chemicals to induce the disease we will be studying. Some of these animals will have the surgery but not have the vital step; these will act as control animals (to show what would have happened if they experienced all the steps without inducing the disease). Surgery is performed under anaesthesia (the animals will be asleep for the duration of the surgery and will feel no pain). Recovery from the types of surgery in this licence should be unremarkable. Most importantly, anything administered or surgery to induce disease should only cause minor impairments in the way the animal moves, only detectable by special tests.

Most of the animals will undergo some tests to assess how well they can coordinate their movements to complete tasks such as grasping for a treat, or removing a piece of tape from the top of their paw. Sometimes, tests looking at more general movements, such as grip strength, balance, and gait analysis (how the animal walks) will be used. These tests will generally not cause any stress or discomfort.

Sometimes, taking blood samples may be necessary to look at levels of indicators of disease (biomarkers) or how much drug is in the blood. Blood samples are usually small in volume and are taken from a superficial (near the skin surface) vein.

At the end of the studies, most animals will be put under terminal anaesthesia (made unconscious permanently) while a large blood sample is taken, and a stain or fix is injected to mark the brain for further analysis ex vivo (in the lab). The animals will be humanely killed while still under anaesthesia.

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

In most cases neurodegeneration will be induced under anaesthesia (while unconscious) if surgery is needed to block blood vessels or give chemicals directly into the brain. Sometimes chemicals may be administered into the body through common injection routes previously detailed (only transient discomfort) if it is known to reach the brain. Stroke will always be induced under anaesthesia.

The symptoms felt by inducing neurodegeneration or stroke, after recovery from any surgery, will be very minimal, and should only be noticeable by special tests. Some of these tests may cause temporary anxiety while the animals get used them, but overall the tests will not cause any lasting harm.

Sometimes the chemicals used to induce neurodegeneration might cause abnormal movements, but these are expected to be mild and will not cause lasting stress to the animal.

The test drugs given are not expected to cause lasting harm, although sometimes the animal may lose some body weight while adjusting to them. Blood sampling is not expected to cause any lasting discomfort.

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

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

Mice:

Mild 35%

Moderate 65% Rats:
Mild 20%

Moderate 80%

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

- Killed

Replacement

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

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

The work to be performed in this project cannot be fully replaced by using models in a laboratory. Wherever possible, work is performed either using cells (a large proportion of a typical work program will be done in tests done with cells grown in the lab) or tissues taken from a dead animal before moving into live animal studies. This is to make sure that the animal studies are well designed and that the maximum amount of data is gained from each animal study.

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

Our company regularly uses a range of in vitro (taking place in a test tube in the laboratory dish) methods utilising cells to understand how a novel test agent might affect the cellular functioning of those cells. From these experiments we can prioritise test agents and only take forward those that have the desired effect, and therefore those that look the most promising for the treatment of neurological disorders.

Why were they not suitable?

Cell-based methods are useful to test the impact that novel test agents have on a cellular level, for one particular type of cell, but they do not model how cells interact with other types of cells and organs, which is particularly important with how brain cells communicate. In addition, cell-based methods do not test the effects that the body might have on a test agent, for example, how it is absorbed, distributed and removed from the body, all of which can alter how effective the test agent might be. Testing a new drug in a well-understood, whole biological system enables us to study the complex interaction in the body, as well as monitoring how the test agent performs in a whole animal. None of the

alternatives mentioned can replicate this, though cell-based testing enables us to prioritise drugs and only take the best candidates forward for further 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 number of animals to be used has been estimated by analysis of the number of animals used on previous projects, looking at the number of animals required for each type of study. This was then combined with a prediction of likely demand of future projects to give the numbers in this project.

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

We have extensive experience in the design of experiments of the types in this project, which has given us confidence in the number of animals required to ensure that no animals are used unnecessarily, but also that the data generated is reliable.

We regularly refer to the PREPARE and ARRIVE guidelines and make use of the NC3Rs Experimental Design Assistant to ensure that we are using the correct number of animals for every study.

Where information is not available in published literature or from contact with other researchers, pilot studies in a small number of animals will be used first where appropriate to assess the action of test agents, and well as the variability in replicating the disease.

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

Data from pilot studies and previous experience are used to ensure that the numbers used are both as low as possible, but also large enough to generate reliable data. Within our company, a member of the wider team has generated a tool for performing power calculations (a way of telling whether the number of animals in a study is enough to produce data that can be trusted) and can be consulted as necessary to assist with study design.

Sometimes animals are produced on our breeding project that don't have mutations (wild-type) that would be suitable for this project, so these animals will be used whenever possible. This will reduce the need for obtaining animals externally. Where animals are obtained from external sources, only the number of animals required for the study will be purchased or imported.

Wherever possible, our in vivo (animal work) scientists will be blinded to the treatment status or even which animal has had which kind of surgery, reducing bias. This enables more reliable information to be gathered from a smaller number of animals.

Baseline data (e.g. bodyweight) are recorded and animals spread across treatment groups to ensure there are no differences between the groups at the start of the study (pseudo-randomisation).

Good planning ensures that within any series of studies we can control for variability that might be introduced. To limit this variability we look at using animals of a similar age/weight range, testing different batches of test agent in the lab first, using the same source of reagents (chemicals used during the experiments), keeping records of observations made and standardising as many components of an in vivo model as is possible.

Refinement

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

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

Animals will be housed in a purpose-built, state-of-the-art facility, that is specific pathogen free (SPF), which excludes certain disease-causing organisms such as bacteria, viruses and parasites that would compromise the health of the animals and the quality of the study output (how good the data is). They will have access to food, water and items that enhance their environment, such as tunnels, chew sticks and mezzanine levels to climb on. Our company staff and the animal care staff are competent in rodent welfare and will ensure that animal suffering is minimised. We aim to house mice and rats in social groups to promote normal behaviour. However, aggressive behaviour can occasionally result in animals being singly housed to prevent injury.

All animals will be wild-type; this is because the human diseases will be mimicked by making changes to the brain, either with changing blood flow to different areas, or by giving chemicals that change how the brain functions.

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

Adult rodents such as rats and mice are the lowest species of mammal that allow us to adequately study the complexities of human neurological disorders. Mice are used wherever possible for studies not involving behavioural tests more suited to rats. Due to the need to conduct simple behavioural tests to assess how the animals move and behave, terminal anaesthesia is not a possibility. It is also important that we are able to monitor the behaviour of the animals both after the neurological disease is induced, and while giving test agents as this allows us to monitor for adverse reactions.

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

Husbandry (animal care) after procedures involving surgery is very important; we will make sure that the animals are kept as comfortable as possible after surgery, and will

include pain relief, soft bedding, and a warm cage to help recovery. In-depth daily monitoring of body weights and general condition will be in place for approximately 10 days after surgery. Where possible, pain relief will be given by putting it into a palatable food like Nutella; this means that the animals will take the medication by choice rather than an injection.

When blood sampling or dosing, we will use the smallest needle size possible to minimise any pain and distress to the animal. We also give drugs via drinking water instead of by mouth where possible.

Where it is hard to predict how a drug will affect an animal, a tolerability or safety study will be performed beforehand on our related project licence to ensure that the drug is tolerable for long-term dosing in our surgical animals.

When testing how the animals move, they will be trained to get used to the tests before data is recorded. This reduces anxiety over the course of the tests. Likewise, animals will be acclimatised to (allowed to get used to) new environments and handling before the studies start.

The choice of strain of rat or mouse will be considered carefully for each study. Based on published work and our own experience, we will consider how each strain is more or less susceptible to different chemicals or potential treatments. Likewise, we will consider the known behavioural characteristics and husbandry needs of the strain; for example, how easy it is to train a rat to perform the behavioural tests because they are naturally less stressed.

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

We follow the PREPARE guidelines for the planning of studies (Smith et al., PREPARE: guidelines for planning animal research and testing. 2020. Laboratory Animals). LASA (Laboratory Animal Science Association) also has a range of published guidance documents with principles that can be applied to our animal studies which are found at https://www.lasa.co.uk/current_publications/.

Specific to ischemic stroke, we will refer to the IMPROVE guidelines where possible. Sert NP et al (2017) The IMPROVE Guidelines (Ischaemia Models: Procedural Refinements Of in Vivo Experiments). JCBFM 37(11): 3488-3517. doi: 10.1177/0271678X17709185.In addition we will also refer to the following when conducting our work:

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. (E Lilley and M. Berdoy eds.). http://www.lasa.co.uk/publications/

Smith D, Anderson D , Degryse A, Bol C, Criado A, Ferrara A, Franco NH, Gyertyan I, Orellana JM, Ostergaard G, Varga O, Voipio H (2018) Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Animal 51(1S): 5-57. doi: 10.1177/0023677217744587

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

When designing animal studies we consider the appropriate guidelines, including the guidance from LASA, the NC3Rs, and the PREPARE guidelines. This guidance will influence our study design.

24. Treating diabetic retinopathy through targeting mitochondria

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, therapy, mitochondria, retinopathy, inflammation

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's the aim of this project?

There is evidence to suggest that by enhancing the replacement of damaged mitochondria (the small batteries that produce energy inside the cells) that sight loss associated with diabetes can be reduced. The aim of this PPL is to understand how this works, and identify drugs that can be used for this purpose.

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

Why is it important to undertake this work?

Diabetes is an overwhelming and increasing public health problem, with 415 million people affected world-wide, and projected to rise by 35% within the next 25 years. Diabetic retinopathy (DR) comprises one of the most common complications, affecting ~80% of people who have had diabetes for 1-2 decades, and is driven by numerous insults in addition to high-blood sugar, importantly systemic inflammation. Even with optimal control

of risk factors (e.g. blood-sugar levels), the incidence of DR remains very high, and is aggravated by the lack of effective and safe treatment. Given the deterioration of mitochondria in diabetes, including the eye (causing DR) and immune system (causing inflammation that exacerbates DR), developing new therapeutics capable to replace the damaged mitochondria may offer innovative solutions to fight against this devastating visual condition.

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

Based on promising data, we want to further understand whether drugs capable of replacing damaged mitochondria can help prevent eye damage commonly seen in diabetes. Therefore, by the end of the project we expect to:

Understand how mitochondrial damage contributes to diabetic retinopathy and in the systemic, low- grade chronic inflammation that contributes to its progression.

Develop and evaluate the potential of novel mitochondrial therapies to treat diabetic retinopathy in animal models.

Inform the scientific community and the general public (e.g. diabetic patients) about the potential of these experimental therapies, through patient and public events, conferences and high impact peer reviewed publications.

Therefore, the information gained from this project may lead to the development of potential therapies to treat DR.

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

This project will enhance our basic understanding to Diabetic Retinopathy (DR) and how its progression is influenced by the chronic low-grade inflammation that accompanies this condition. We aim to alleviate DR by targeting the damaged mitochondria that accumulate in the retina and in the immune system, so demonstrating the preclinical efficacy of our therapies is a key step to enhance their implementation in the clinic. Therefore, this research has the potential to significantly reduce the clinical, economic and patient burdens associated with diabetic retinopathy (DR). In the UK, around 2% of people who have had diabetes for 1-2 decades have DR, which poses a significant emotional and physical burden for patients.

Short-term (within the five-year project): the beneficiaries will be the local, national and international academic communities involved in diabetes, retinopathy and mitochondrial-based therapies. Benefits will include both scientific discovery and technical expertise, which can be shared through conferences, meetings, publications and social media. Also in the short term, patients will be aware of our work through focus groups on diabetes and eye disease (this is currently running and will expand in the upcoming years).

Mid-term (5-10 years after this project): This project will determine the potential of new therapies to prevent eye damage which often occurs in diabetes (diabetic retinopathy). Such therapies showed promise in our preliminary studies, including improved visual function. Our multi-disciplinary team (scientists, doctors and pharmacologists) has good engagement with hospitals, patients and pharmaceutical companies who will help bring the treatment to diabetic patients. In addition, we will actively seek pharmaceutical partners who are currently developing drugs with a similar mechanism for application in other diseases, thus accelerating the path to implement our technology towards essential phase-

III trials.

Long-term (10 years after this project): Through knowledge acquisition and therapeutic discovery, patients and clinicians will be the most likely beneficiaries to this research. For diabetic patients, it will be the development of a treatment which alleviates sight loss, administered in a way that is patient acceptable (orally) and improve current practice in diabetes care. For clinicians, the availability of new oral therapies able to alleviate diabetic retinopathy, would involve a revolutionary approach.

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

Knowledge sharing of positive and negative results and approaches: results from this project will be important for advancing our knowledge in diabetes pathology, new disease targets and any new treatments. The results of the models, techniques and data will be published in peer-reviewed journals to reach a wide audience (e.g. ophthalmology, diabetes care, mitochondrial biology). Any negative data will be addressed and described in order to allow others to learn and optimise methods. Social media and dedicated magazines for the general public will also be used to promote the research and its results.

Our group will maximize the results of this research locally, by banking a large number of diabetic tissues for future studies and also by sharing the surplus animal tissues with other colleagues. We will also maximize outputs nationally and internationally, by fostering collaborations, disseminating new knowledge, and publishing results and methods to inform the scientific community.

Collaborations: We have established a number of collaborations to investigate pharmacodynamics and the molecular targets of our mitochondrial therapies. This will enhance the translation of our therapies into diabetes care.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

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

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

Mouse models of diabetes have been extensively investigated and routinely used for vision research. Their ocular anatomy and functioning is very comparable to the human eye, and therefore is an ideal surrogate to model the human disease. In addition, mice also recapitulate the underlying pathology of human diabetes, including hyperglycemia and systemic inflammation that contribute to the development of diabetic retinopathy from juvenile ages. Therefore, mice that develop spontaneous diabetes in young stages (4 weeks of age) have been chosen for this project.
Typically, what will be done to an animal used in your project?

Typically, by 1-2 months of diabetes, hyperglycaemia and inflammation will be assessed in mice, using small volumes of blood. Animals will be then treated with oral therapies (in the drinking water) for long- term (until 8-9 months of diabetes). During the treatment, animals

will have their blood withdrawn on occasion (e.g. monthly), to evaluate the effect of the therapy in relieving inflammation that contributes to diabetic retinopathy. Mice may undergo procedures under a final anaesthetic from which they will not recover: (i) non-invasive imaging of their eyes performed, using optical coherence tomography, and/or injected with a label to better allow post-mortem evaluation of blood vessel in the eye. At the end of the treatment, mice will be humanely killed and the eyes collected for further laboratory investigations, so we can understand the level of eye protection achieved by our therapy. The experiments will typically last for 9-10 month.

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

The mouse model used in this research have decreased pancreatic function and significant hyperglycaemia by 4 weeks of age. As with humans, the progression of diabetes may induce adverse effects in some mice, including a reduction in weight gain, polyuria, increased risk of moist dermatitis (urine scalding) and/or rare development of cataracts. Therefore, diabetic mice will be closely monitored and animals will be humanely killed if such adverse side-effects cannot be relieved in the short term. All the therapeutic agents that will be used in this application have undergone extensive testing via other methods, including cultured cells prior to use in living animals to minimise any risk of adverse effects, as well as in preliminary studies using a small number of animals. Nonetheless, mice will be closely monitored for any weight loss or abnormal behaviour for (unlikely) intolerance to the therapeutic agent and humanely killed.

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

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

The protocols have been classified as:

- Mild (33%)

- Moderate (67%)

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

- Killed

Replacement

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

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

Animal use is central to this project because of the complex nature of the pathology that occurs in the eye and that is influenced at the systemic level (e.g. hyperglycaemia and a chronic low-grade systemic inflammation). Eye damage in diabetes arise over a prolonged period and cannot be faithfully reproduced using tissue culture or organ explants. Therefore a fully functioning living system is needed so that we can model whether new

oral mitochondrial therapies can alleviate vision loss and the underlying diabetes pathology affecting this condition.

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

Wherever possible we do seek to use in vitro cell culture models within our laboratory (e.g. retinal and/or vascular cells grown under high glucose). These cellular platforms will be instrumental for identifying drugs capable of replacing damaged mitochondria in the retina, understand their mechanism of action, identify their possible adverse effects, and infer doses for the in vivo studies.

Why were they not suitable?

We want to understand whether drugs that replace damaged mitochondria can help prevent damage to the eye that occurs in diabetes (using treatments given in the drinking water). Whilst non-animal alternatives will be essential to identify suitable drugs and understand their precise mechanism of action, diabetic mice will be essential to understand whether our therapies work at a systemic level.

Reduction

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

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

The numbers estimated in this project are based upon 9 years of experience of experimental design and from previous experience of using the same diabetic model in previous PPLs. Most of the experiments have quantitative end points, and my previous experiments and/or published data have demonstrated that we require around 8-9 animals per treatment group in order to develop statistically sound results. Where these data is not available, we will perform pilot studies based on our experience to provide preliminary data for these calculations.

Although we will reduce the variability as much as possible, some diabetic mice have inherent differences in the development of retinal complications and, like humans, may present a less pronounced phenotype.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?
Our primary goal is to monitor the effects of such therapies to improve diabetic retinopathy and its underlying pathology (e.g. systemic inflammation). To avoid experimental biases that could jeopardize the validity of the results, animals will be assigned to different experimental groups randomly. We will only use drugs in vivo where in vitro approaches have indicated that they can successfully replace damaged mitochondria (using an innovative cellular platform).

All experiments will be hypothesis driven with clear end points to help aid the design. Study designs are checked against ARRIVE2.0 guidelines. The EDA tool will be used to

minimise any design error or bias and to ensure that we report the study results comprehensively. We often consult statisticians to help derive the correct powering of the study or to review our experimental designs before the study starts.

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

Initial studies will be designed based on previous experiments. Given that I have over 9 years' experience of using this diabetic model, I am confident in the experimental design for studying retinopathy and the underlying pathologies contributing to its progression (systemic inflammation). All of our therapies capable to replace damaged mitochondria have been administered by oral routes and thus, the experimental design protocols are already in place.

In addition, reduction in animal numbers will be achieved through:

The use of males as the only experimental mice, since the penetrance of diabetes is incomplete in females. Including diabetic females would thus introduce a high degree of experimental variability in our studies.

We will obtain a large number of readouts in each mouse, using non-invasive techniques to assess retinal degeneration and systemic inflammation. By the end of treatments, we will harvest the eyes, to understand if our therapy can protect the retina. This approach will allow us to obtain as much information as possible from each animal to drastically reduce their numbers.

We will use of both eyes to evaluate changes. This will enable eye tissues to be pooled for analysis where needed. We will also use molecular techniques from where we can evaluate retinal health using minimal quantities of tissue.

Since our therapy will be delivered systemically, in addition to the eyes, we will bank a large number of organs to evaluate multisystem therapeutic potential of the drug for future studies (pancreas, heart, kidneys, etc). This will optimise the data and information from all animals in all experiments, by allowing us to understand the potential of our therapy in treating different diabetic 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.

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

The experiments detailed here will be carried out in a mouse model of type-1 diabetes. This model has been rigorously characterised and is suitable for use in diabetic retinopathy research, since it recapitulates both early and late disease signs with equivalent mitochondrial pathology that lead to retinopathy in humans. Whilst chemically

induced models of diabetes (e.g. streptozotocin) are arguably the most common mouse model of diabetes used worldwide, the streptozotocin model displays a more aggressive retinal degeneration that is not representative of early diabetic retinopathy in humans (thus, attributable to the neurotoxic effects of streptozotocin and making it less appropriate for this study).

Another advantage of the chosen mouse model is that unlike many other rodent models of diabetes they exhibit hyperglycaemia and hypoinsulinemia in the absence of insulitis or obesity which might otherwise complicate data interpretation.

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

Less sentient animals such as fish are not suitable for modelling human diabetes and their associated eye and underlying systemic inflammation. Unlike mammals, they are able to regenerate their injured retinae and therefore, will not be suitable to answer our scientific questions. In addition, the use of embryonic and terminal only procedures are not possible, given the need to evaluate the potential of our therapies to alleviate retinopathy and the associated systemic inflammation that slowly develops during diabetes. However, where possible, we will perform measurements at the end of the study under a terminal anaesthetic rather than repeated anaesthetic events.

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

As scientists we are aware of the LASA guidelines and have a strong sense of responsibility for the welfare and care of animals bred for and used within our work. The probability of encountering and adverse outcome will be diminished by planning preliminary studies and by putting in place stringent physiological end point criteria and close monitoring:

We also opted to deliver drugs by the least invasive manner (in the drinking water).

If a new dose for a drug needs to be determined for administration in drinking water, a small preliminary study will be performed to discard any adverse effect (using 2-3 mice).

Welfare of diabetic mice will be optimized by providing more absorbent bedding (due to excessive urination volume).

Animals will be humanely killed in the event of them reaching their humane endpoints with regard to body weight loss, urine scald, and body condition, as judged using a bespoke welfare scoring sheet.

These refinement steps, and those still in progress, will streamline experiments and reduce the use and suffering of animals.

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

The ARRIVE2.0 and PREPARE guidelines will be used to help plan, design and report on the experiments in order to ensure our studies are conducted in the most refined way possible, according to LASA guidelines.

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

advances effectively, during the project?

I will use the NC3Rs website to stay informed of latest developments, together with newsletters from our animal facility. I have previously attended the "UK Home office project licence (Module 5) course, and this knowledge will be key to be pro-actively informed and continuously improve my animal research practice by attending local workshops and engaging in seminars and events given by the NC3Rs (https://nc3rs.org.uk/webinars).

25. Leucocyte Trafficking 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

Immunology, Leukocyte trafficking, autoimmunity, cancer, therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to understand the basic mechanisms of leukocyte migration and how these are altered during disease. Importantly, we aim to develop strategies to correct leukocyte trafficking as novel therapeutic tools for human diseases and test these strategies in relevant models of disease to allow their fast progression to clinical use.

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

Why is it important to undertake this work?

New therapies are needed to control and manipulate unwanted immune responses in multiple diseases such as autoimmunity, cancer and transplant rejection. Leukocyte migration is a key step of immunity.

Understanding and comparing the mechanisms that regulate leukocyte trafficking in health and disease will help identify new targets for innovative therapeutics - as we have successfully done in the last 5 years of tenure of the project licence.

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

We are confident that this project will create new knowledge (in the form of communications at meetings, through publications and data sharing), leading to new therapies for diseases in which these are lacking, for example myocarditis. The focus on metabolism will allow the repurposing of existing drugs, developed for other or similar diseases, as we have done successfully in the past (see Kishore et al. Immunity 2017).

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

The primary potential benefit of this project relates to new knowledge about the mechanistic regulation of leukocyte migration in health and disease. This study will generate numerous research outputs all of which will be disseminated in the form of publicly available open access research papers, research datasets, new methodology and reagents. The data generated will also aid developing experimental refinements to improve animal welfare and refine the experimental design by identifying confounding factors or uncontrolled variables to improve reproducibility. The information will be of interest to basic immunologists.

The secondary key benefit relates to the translational value of the results to clinicians, particularly in the field of immunotherapy, and to the possibility that new molecular targets may be identified, for which new drugs could be developed for a variety of human diseases in which inflammation plays a major part, from cancer to transplant rejection. These treatments will be able to be applied to patients after further pre-clinical studies. We believe that these are likely to improve survival and quality of life of a large number of patients. Treatment costs for these patients may also be reduced.

The studies will be relevant to diseases which have a high (diabetes, pre-eclampsia, cancer) prevalence in the populations, as well as helping the diagnosis of diseases, such as myocarditis, whose frequency has been underestimated as many patients are asymptomatic during the acute phase of disease and present years later with acute or chronic heart failure. Survival of transplanted organs, as well as patients bearing a transplant, has not improved in the last 20 years, due to imperfect knowledge of the mechanisms of immune cell migration to the graft as well as the severe side-effects attached to conventional immunosuppression (many patients die from infections and cancers). We aim to provide new knowledge and, subsequently, new therapies to prevent transplant rejection by selectively preventing migration of immune cells to the graft, while preserving the rest of the immune system (hence reducing severe side effects).

The success of the proposed project strategy is underpinned by the identification, in the previous tenure of the license, of a drug that induce migration of anti-inflammatory, regulatory T cells to inflamed tissue, thus resolving inflammation. As a result of these observations 2 clinical trials are already on-going.

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

The outputs of this project will be maximised by dissemination of results, datasets and methodology in open access, which will be easily available to the research community internationally.

The translational value of this study will be maximised by parallel clinical studies conducted in vitro with blood and tissue samples from patients. This will allow selecting mechanisms and therapeutic targets, which can swiftly translate to clinical settings.

Collectively, our approach will lead to new therapies, attach further prestige to UK Academia and create new jobs in science for outstanding individuals.

All these outputs have substantial economic benefits for the UK.

Species and numbers of animals expected to be used

- Mice: 9800

Predicted harms

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

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

Rodent models are typically used in immunological research. A large body of literature exists on leukocyte trafficking in mice, which will avoid the need to reproduce/develop well established findings. In addition, a plethora of reagents relevant to this project are commercially available with well-defined dosing for in vivo studies. Finally, genetically altered strains with lack or modification of molecules relevant to this project are already available, thus reducing the need to generate new genetically altered animals. We will use animals in which sex and life stage are relevant to human disease (e.g. adult male mice in Myocarditis).

Similarly, validation in robust preclinical models of disease is required for the progression of potential therapeutic strategies into clinical trials. All the disease models in this project have been selected based on their extensive use to validate the effect of drugs that are now used in human disease.

The life stage and sex of the animals has also been chosen based on the relevance to the human diseases under investigation.

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

Typically, an inflammatory process will be induced in animals in order to study the role of selected molecules involved in leukocyte trafficking and the effect of modulation of these molecules in inflammation.

Inflammatory and disease models will include administration of pro-inflammatory substances or cells, skin or heart grafts, myocarditis and tumours.
In some of these animals the immune system might be modified prior to the induction of inflammation by using recipient animals with altered expression of molecules of interest, or disease-prone and pregnant animals (type I diabetes and pre-eclampsia respectively), or by bone marrow replacement to modify immune cells. In the latter instance, we will generate mice bearing a modified molecule of interest only expressed by hematopoietic stem cells (HSC i.e. leukocytes) from GA animals with tissue-non-selective modification or

by in vitro genetic modification of the HSC (using for example shRNA transfection) when appropriate GA strains are not available or are not leukocyte-selective.

After induction of inflammation, animals will be treated with drugs that modify leukocyte migration or with cells that are made to migrate better by ex-vivo treatment to reduce inflammation or, in tumours, increase inflammation and tumour rejection.

Of note, our experimental endpoint is the measurement of leukocyte migration which occurs at early stages of the inflammatory process, hence in most of the protocols animals will be humanely killed prior to full disease development, thus avoiding unnecessary suffering.

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

The proposed experimental models have been chosen based on their relevance to human diseases and the least possible suffering for the animals. Except Protocol 1 (mild), all Protocols are of moderate severity.

We have proposed this level of severity based on careful refinement of the protocols to substantially reduce animal suffering, while enabling the delivery of highly informative and translationally relevant data.

For example, in the myocarditis model, which involves the use of Freund's adjuvant, optimising the injection site in an area where local pathological responses cause least pain (based on animals' behaviour) and the introduction of analgesia, has substantially reduced the severity of this model.

All transplants in the mouse will be performed in a dedicated operating suite by skilled personal license holders according to strict Home Office, Institutional and laboratory guidelines and kept under constant review by an institutional review panel and institutional veterinarians. Anesthesia, analgesia and post- operative recovery protocols have been developed in close consultation with the institutional veterinary service and represent current best-practice to minimize distress. Recovery from surgery is closely monitored and importantly, animals return to normal patterns of behaviour (movement, feeding, drinking) within twelve hours of surgery.
Monitoring of transplant outcome requires a simple visual inspection of the transplant (skin grafts), or finger-tip evaluation of transplant function by palpation (heart grafts).

In the skin graft model, animal movement will be partly restrained by application of a cast to prevent them from scratching the graft and causing infection. We have refined this model in such a way that the cast is kept for no longer than 7-8 days and the movement restraint is minimal by cutting the cast appropriately based on the behaviour/mobility of each of the experimental animals.

In the cancer model, our studies will focus on early stages of solid tumour development. The cancer model we propose will involve injection of non-metastatic tumour cell lines in subcutaneous tissue left to grow locally and investigate the mechanisms by which immune cells that can destroy early tumours are excluded thus allowing the tumour to progress. From pilot experiments under the current project license, we have established that a tumour of approximately 5 mm diameter is sufficient for analysis of infiltrating leukocytes. Animals bearing tumours of this size in the subcutaneous tissue do not display any sign of suffering or altered behaviour.

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

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

Except Protocol 1 (mild), all Protocols are classified as moderate.

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

- Killed

Replacement

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

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

The plan is to identify key molecules that regulate leukocyte migration. This involves a lot of preliminary in-vitro laboratory work, using tissue culture techniques. Migration of leukocytes, however, is an extremely complex process, which requires the leukocytes to localise to different site of the body by interacting with other cells and crossing complex barriers (vessel walls), in a coordinated manner. Appropriate testing requires a fully formed vascular network, which is not achievable by using computer-based systems, lower organisms and embryo stages, cultured cells, tissue, and organoids. Only living animals can be meaningful models for the purpose.

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

Prior to undertake experiments in animals, we typically conduct a series of in vitro experiment which help to optimise in vivo experiments and reduce the number of animals required. For example, we use flow chambers which can reproduce some of the events which happen during leukocyte migration in vivo.

In addition, we check the availability of existing data sets and/or existing data within the lab that can be used to conduct novel meta-analyses before collecting new data.

Why were they not suitable?

The in vitro models only give a limited amount of information, and cannot faithfully reproduce physiological settings, due to the complexity of leukocyte trafficking events which occur in vivo.

Importantly, pre-clinical validation of therapeutic regimens needs to be done in order to evaluate their effects and potential off-target effects prior to- and as a condition of- their use in 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?

The relatively large number is due to the inclusion of genetically altered animals, which are extremely useful to clarify the role of specific cells or molecules in the regulation of lymphocyte trafficking. These mice need to be bred for several generations before they are suitable for experimentation so a large part of them (40%) will not undergo more than one procedure (breeding). In addition, my lab includes 15 researchers, of whom 12 work with experimental models.

A minimum colony size sufficient to generate animals for our investigations will be maintained for each strain. With advice from our NACWO we monitor animal numbers and adjust breeding programmes accordingly. We carefully consider minimal group sizes, number of groups to be studied, use of one or both sexes of animals as appropriate and optimisation of protocols. Small-scale pilot studies are always conducted to see if larger scale studies are warranted.

The number of mice to be used in each Protocol has been estimated by power calculations to include the minimum number of animals to obtain statistical significance. We regularly input our experimental designs- and run our power calculations using- the NC3Rs EDA tool.

Advice on the proposed experimental designs and methods of analysis of the results will be taken from the Statistical Services Unit. Where relevant, factorial experimental designs will be used, rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum resource. For most of the quantitative 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%.

We are committed to ensuring reduction of animal numbers through appropriate experimental design and looking for alternative approaches and using statistical analysis packages to ensure that we only use the minimum required to generate statistically meaningfuldata.

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

A wide literature search has confirmed that our project is original, and that there is no duplication with previous reports. For quantitative experiments, animal numbers needed are statistically determined using power analysis. We have refined suitable models over the years, which allow using a small number of animals to achieve statistical significance. To assure reproducible outcome, which will maximise the information obtained from the minimum resource, experiments will be carefully designed and performed, including randomisation of treatment or control groups, allocation concealment, and blinded assessment of outcome, and explicit inclusion and exclusion criteria. In addition, tissues from the same animal will be used in as many analyses as possible to minimise the number of animals required.

Of note, the use of bone marrow (BM) replacement, using BM from GA animals or wild-type BM genetically modified in vitro, has allowed substantial reduction of our breeding

programme by approximately 50%, hence this procedure is included as a step in a most protocols.

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

We continuously strive to refine our protocols to minimise animal suffering, but also to reduce the number of mice needed to reach statistical significance. For example, we have been able to increase the incidence of disease in our model of myocarditis from 40 to 70%by optimising the injection site. This means that we need only 50% of the animals to power our experiments.

In breeding of genetically altered animals, efficient colony management will ensure 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 or embryos as the main method of cryopreservation, which reduces the number of animals required to secure a line. We will use good practice in experimental planning, including statistics such as power and resource equations and breeding calculations using current breeding figures to predict the number of matings required for experimental cohorts.

Finally, whenever possible, we will source/purchase animals externally to avoid unnecessary breeding of large number of animals under this licence.

Refinement

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

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

We will use mice mainly of the C57BL/6 strains, which are the most suitable for this large-scale basic and translational study. Importantly, there are a plenty of useful research materials (like antibodies) for mice. General features of leukocyte trafficking in murine models are sufficiently equivalent to humans to obtain translational data. In addition, we will be able to use genetically altered animals, an extremely useful tool to elucidate basic and pathologic mechanisms while reducing procedures on animals (e.g. continuous administration of drugs etc.).

We will breed genetically modified animals which have alterations in molecules of interest involved in leukocyte trafficking. Whenever possible we will use animals in which alterations of the molecule of interest is restricted to leukocytes, and this will avoid potential pathologic phenotypes due to whole- body modifications.

We will use a variety of models that closely reproduce either physiological or pathological leukocyte migration.

For the study of physiological leukocyte migration we will use a model of peritoneal recruitment of leukocytes and one model of intra-lymph node injection, which allow studying leukocyte egression from lymph nodes. In addition, we will study leukocyte migration during pregnancy and angiogenesis. These models have been refined to minimise animal suffering while reducing the number of animals needed for statistical significance. Refinement in these models has been achieved by well-designed pilot studies to reduce the number of animals and procedures in the relevant protocols, by minimising the dose of pro-inflammatory substances to a minimum which allows the measurement of a biological effect without animal suffering, and by using leukocyte migration-eliciting substances with negligible pain- inducing effect (for example, we use chemokines instead of LPS).

For the study of pathological leukocyte migration we will employ established models of human disease, including graft rejection, autoimmunity, dysmetabolism and cancer.

In graft rejection models (including human tissue xenografts), surgical and anaesthetic techniques have been refined. Post-operatively, animals will receive intensive care for several hours in special recovery cages. Post-operative pain will be prevented using analgesics. Infection will be prevented by using aseptic procedures in dedicated surgery and recovery rooms. Dehydration will be prevented by administration of fluids and minimal blood collection. To minimise mortality due to surgical procedures, cardiac grafting will be exclusively conducted by a permanent member of the group who is extremely skilled in microsurgery. In skin grafting, impaired mobility of animals due to Paris Cast has been minimized by optimising the cutting of the areas around the animal limbs as well as the use of Jellonet under the casts.

Experimental autoimmune myocarditis closely resembles human myocarditis (histopathology, sex bias, autoantigen etc.). We have refined our technique to minimise animal suffering by identifying an injection site where local pathological responses cause the least pain (based on close monitoring of the animals), and can be readily observed and treated if necessary. Based on a pilot study we are also going to introduce analgesia, as we have observed that this intervention does not interfere with the induction of autoimmunity.

In the dysmetabolism model, we have introduced an acclimation step prior to transferring the animals to metabolic chambers, which has substantially reduced sings of stress in the animals.
Finally, we have refined the model to study leukocyte migration to cancer so that we can conduct our analyses when the tumour (subcutaneous) has reached a mean diameter of 0.5cm, a size that does not normally lead to tumour ulceration, movement reduction, or any sign of cachexia. Further, we have selected non-metastatic tumour cell lines for this study. This refinement has also improved the quality of our data, as tissue necrosis associated with advanced tumours also affects infiltrating leukocytes.

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

Less sentient species are not suitable for the project, due to to the physiological and genetic distance from humans, the lack of suitable reagents for investigation and of relevant genetically altered models.

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

In the skin graft model, animal movement will be in part restrained by application of a cast to prevent them from scratching or chewing the graft and causing infection. We have refined this model in such a way that the cast is kept for no longer than 7-8 days and the movement restraint is minimised by keeping the animals' limb areas totally unrestricted.

The cancer model will involve injection of tumour cells under in subcutaneous tissue to grow locally. From pilot experiments under the current project license, we have established that a tumour of approximately 5 mm diameter is sufficient for analysis of infiltrating leukocytes. Animals bearing tumours of this size in the subcutaneous tissue do not display any sign of suffering or altered behaviour. Whenever possible, we will use tumour cell lines that do not spread by metastasis, hence further reducing potential suffering.

Surgical and anaesthetic techniques have been refined. Post-operatively, animals will receive intensive care for several hours in special recovery cages. Post-operative pain will be prevented using analgesics. Infection will be prevented by aseptic procedures in dedicated surgery and recovery rooms. Dehydration will be prevented by administration of fluids and limited blood collection. In an unlikely event that animals do not recover from surgery well or develop unexpected complications, they will be humanely killed.

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

We will refer to the following:

Guidelines for the welfare and use of animals in cancer research - https://www.nature.com/articles/6605642

A guide to defining and implementing protocols for the welfare assessment of laboratory animals: eleventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement - https://doi.org/10.1258%2Fla.2010.010031

Refining procedures for the administration of substances Report of the BVAAWF=FRAME=RSPCA=UFAW Joint Working Group on Refinement - https://journals.sagepub.com/doi/pdf/10.1258/0023677011911345

Appendix 4 HO Minimum Standards for Aseptic Surgery.

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. http://www.lasa.co.uk/wp- content/uploads/2017/04/Aseptic-surgery-final.pdf

PREPARE guidelines. https://pubmed.ncbi.nlm.nih.gov/28771074/ ARRIVE guidelines for reporting. https://arriveguidelines.org

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

Our NACWO/NIO regularly distributes updates from the HO and NC3Rs to all personal and project licence holders.

In addition, we regularly check the https://www.nc3rs.org.uk for updates on replacement, reduction and refinement.

Any new refinement relevant to this project will be discussed with the NVS and implemented whenever possible.

26. Adaption and pathogenicity of bacterial pathogens at the host interface

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

bacteria, infection, host adaptation, colonisation, therapeutics

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this work is to understand how bacteria adapt to distinct locations around the body, termed ‘niches’. This knowledge is essential for understanding how pathogens colonise and persist within a host. Furthermore, this information can be exploited to design targetted therapies against infection, such as the development of drugs that inhibit the infection processes or identification of vaccine 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?

Antimicrobial resistance (AMR) is the process of a microorganism changing over time, ultimately resulting in a lack of response to medical treatments therefore rendering such drugs ineffective. AMR is spreading throughout the community and clinic, and is a global threat to human health. The Medical Research Council has estimated that without action

the annual global deaths related to AMR will increase from ~1.4 million currently to ~10 million by 2050 and there are currently very few new drugs in development. This highlights the urgent need to understand how prevalent pathogens cause infection and develop new treatment strategies to overcome AMR.

The bacterial species Escherichia coli is formed of both harmless and infectious types (infectious types termed pathotypes). E. coli pathotypes are capable of causing a wide range of illnesses including severe diarrhoea, urinary tract infections and infection of the bloodstream. Treatment of E. coli infections is very challenging due to the increasing spread of AMR, particularly in E. coli recovered from patients presenting with bloodstream infections. These “extraintestinal pathogenic E. coli” (ExPEC) isolates are often resistant to multiple drugs. This is worrying as infection of the bloodstream is a precursor to potentially lethal sepsis, which affects ~50 million people worldwide each year with a one in five chance of death. E. coli is also the leading cause of bloodstream infection and responsible for ~300,000 AMR related deaths annually.

One strategy to overcome AMR is to design alternative treatments that block specific bacterial functions. To do this, we must first understand how pathogens function at the locations around the body where they infect. Laboratory studies of infection are inherently limited in scope because bacterial behaviour is controlled in response to the their environment. Locations of infection are unique with respect to both the structure of the organ as well as the molecular composition of the environment. It is currently impossible to recreate this complexity artificially in the laboratory, therefore making animal models of infection hugely advantageous in studying details of infection that would otherwise not be identified in the laboratory.

Molecular analyses of genetic material isolated from the precise location of infection (such as gene expression levels) can reveal important information about how pathogens are causing the infection. The importance of specific genes identified by this approach can then be confirmed through the testing of modified bacteria lacking these genes in their ability to infect animals. Therefore, genes that contribute to the pathogens infectious capacity represent potential targets for drug or vaccine development.

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

By the end of the project, we will have developed a clearer understanding of how the bacterial pathogens E. coli and C. rodentium adapt to distinct locations within the host and cause infection therein. This will enhance our basic understanding of bacterial disease by identifying the most important genes involved in the infection process. In addition, the research has the potential to identify drug or vaccine targets in the process. This knowledge will be shared with the scientific community through publication in peer reviewed journals, presentation at national and international specialist conferences, and through public engagement activities

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

What will benefit:

This project will contribute to our basic of knowledge bacterial infection by providing a deeper understanding of the fundamental mechanisms used by specific pathogens to infect the host.

Who will benefit:

In the short term, this research will benefit the infection biology community. The findings will be used to expand our understanding of host-pathogen interaction by identifying the key genes that are regulated in response to distinct host sites. This novel information will be disseminated to the research community through publication and presentation at scientific meetings. Some of our data will be archived in a publicly accessible repository to facilitate colleagues in the field taking advantage of the resource.

In the long term, this work will increase our fundamental knowledge of infection mechanisms used by important human bacterial pathogens and will potentially reveal therapeutic targets that can be exploited in future studies, possibly leading to future treatments for human patients

How will these benefits be realised:

The findings will be disseminated throughout the scientific community via publication in peer reviewed journals and presentation at national and international specialist conferences. Additionally, the findings will be communicated to the public through engagement activities to raise awareness of bacterial infections and AMR. Any relevant data derived from the findings will be shared upon publication of the work, via the most appropriate public repository.

When will these benefits be achieved:

Specific outputs relating to the role of key genes in niche adaptation is possible within the lifetime of this project. The identification and development of viable therapeutic targets will likely take much longer than the lifetime of this project.

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

In the past I have benefitted immensely from academic collaboration. I will therefore offer expertise in the models described here in the form of collaborations to address specific scientific questions related to infection. I will also offer advice and guidance to other colleagues wishing to pursue their own project application adapting the models described within this project.

My methods have been described in recent publications and I will continue to disseminate research related to my existing and newly developed models in the form of peer reviewed papers. Additionally, further dissemination of the research outcomes will be made more widely by presentation of the findings at national and international scientific meetings.

Species and numbers of animals expected to be used

- Mice: 450

Predicted harms

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

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

Adult mice around 8-10 weeks of age will be used in this project as they are fully mature and have an intact immune system capable of responding to bacterial pathogens. Mice will allow the investigation of dynamic and specific interactions that occur between specific pathogens and the host, in real time.
Mouse models offer parallels to human infection, such as infection of specific body sites, that allows us to investigate key steps in the infectious process. This can ultimately lead to the identification of novel therapeutics, which can be tested directly in the same model for an ability to limit host-pathogen interaction. There is a significant body of literature available related to the mouse immune system and response to infection. Furthermore, the protocols described in this project are well established in the literature. Collectively, this makes mice the best model for studying bacterial interactions with the host in the context of pathogen adaptation.

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

Animals will be housed using standard husbandry prior to any infection experiments. Prior to infection, animals may be subjected to a course of antibiotics via the most appropriate route to eliminate the commensal microbiota and may be maintained on this treatment for the duration of the protocol.
Animals will be infected with the stated bacterial species and at the stated timepoint by either oral gavage (O.G.) thereby directly introducing the organism into the stomach (maximum of two occasions) or by intravenous (I.V.) injection into the bloodstream via the tail vein of the mouse. Monitoring of infection will be performed by sampling bacteria from the faeces of infected animals. Alternatively, infection may be monitored by use of the in vivo imaging system (IVIS) whereby animals will be subjected to general anaesthesia and imaged using the IVIS at most twice on the first day and once daily thereafter. The final experience of the animals will be humane culling by a Schedule 1 method.

The three protocols used in this project and their expected outcomes are all well established in the literature and supported by extensive pilot studies performed in a previous PPL holders lab.

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

For single or multiple O.G. inoculations, the impact on animals is largely limited to the stress associated with the procedures used to infect the mice. This will be minimised by employing low stress handling techniques and ensuring the users are competency trained in the O.G. procedure. The animals are not expected to experience any lasting adverse effects such as pain or lasting suffering as a result of the procedures. The infections are self-limiting, but mice may experience some symptoms such as mild weight loss or mild diarrhoea.

For I.V. infections, animals may experience some minor stress and pain due to the injection used to infect the mice. This will be minimised by employing low stress handling techniques, heating of animals to dilate the tail vain and use of a micro sized insulin needles. All animals are expected to experience some mild symptoms such as weight loss, reduced movement, hunching, piloerection and partially open eyes due to the acute infection of bacteria into the bloodstream. It is possible that some animals may experience more moderate symptoms. However, these symptoms are expected to peak within the first 24 hours and resolve thereafter and it is expected that these symptoms will not extend beyond mild-moderate in measure. In longer experiments, the multiplication of the bacteria within the animal could lead to more prolonged symptoms beyond 24 hours but current

data suggests it is not expected that they would increase in measure and it is likely that symptoms will resolve.

For IVIS scanning, the impact on animals is largely limited to the stress associated with the procedures used to anaesthetize the mice and the cumulative nature of multiple exposures. This will be minimised by employing low stress handling during the procedure and ensuring users are fully trained and competent in anaesthetic procedures, using the shortest duration possible.

Careful monitoring in accordance with detailed score sheets will be employed to cover all expected and potential impacts and adverse effects. For all protocols, procedural stress will be minimised throughout by ensuring good technique is used. This will be facilitated through competency training for all procedures delivered by staff within the PPL holders animal unit.

Expected severity categories and the 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 O.G. and I.V. infections, >95 % of animals will experience mild discomfort likely to be associated with the procedures used and < 5% of animals may experience moderate symptoms as a result of the infection. Careful monitoring in accordance with detailed score sheets will be employed to monitor all expected or potential symptoms and will limit the suffering of any individual animal to, at most, moderate discomfort.

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?

Infection is a dynamic process in which disease outcome is often dependent on several factors including the intrinsic responses of both organism and host to one another, the response of bacteria to specific environmental conditions at different body sites or metabolic/dietary status. Whilst some alternatives (including laboratory grown human cells or tissue models) are able to replace the need for whole animals in the study of specific aspects of infection, such as tissue invasion, there is currently no model which reflects the complexity of challenges, stated above, that bacteria encounter during growth within and colonisation of a host. Specific questions related to infection will be addressed in the laboratory as a first approach wherever possible.

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

It is possible to make use of non-animal alternatives in this project to address specific scientific questions. For example, we can use genetics to test the impact that specific gene

deletions have on the ability of specific E. coli strains to express virulence factors or use cultured eukaryotic cell lines to test the ability of said mutants to bind to host cells. Using this approach, we have identified several metabolic genes that impact on virulence factor expression in the gut pathogen enterohaemorrhagic E. coli (EHEC) via metabolism of nutrients that can be encountered in vivo. The use of cultured cell lines will always be employed as a pre-requisite to studies involving mice to ensure that only the most likely genes to be involved in infection are tested in vivo. Additionally, cultured macrophages can be used to study immune evasion mechanisms by extraintestinal pathogenic E. coli (ExPEC) for the investigation of specific genes.

New alternative laboratory-based systems for modelling infection processes are also in development. For example, organoid based human epithelial cell culture systems are currently in development that recreate the oxygen gradient naturally found in vivo at the epithelial cell surface and are capable of culturing anaerobic gut microbes. Using this system, it may be possible to test if EHEC interaction and competition with gut bacteria can be studied using this ex vivo model.

The use of these models, in combination with our increasing knowledge of pathogen adaption to the site of infection, will offer further opportunities to recreate in vivo conditions within the laboratory, potentially reducing the need for animals in this type of research in the future.

Why were they not suitable?

While cell culture models can offer insight into specific aspects of infection, they cannot reproduce the dynamic and ongoing process of infection at distinct body sites, interaction with the microbiota or immune response. Thus, in order to determine the importance of individual pathogen genes for infection, modelling using whole animals must be employed for the most promising targets. Organoids offer a promising alternative that may potentially be able to recapitulate such complex interactions in the future, however the use of these systems is still very much in its infancy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is based on the programme of work described and existing experience of the minimal number of animals needed in each experimental group to provide statistically robust outcomes. Group sizes are constantly being re-evaluated and updated when necessary. In situations in which new mutant bacterial strains, or modification to methods are used, work will initially be limited to small numbers of animals, with several sequential experiments undertaken to achieve publishable data. This will allow measurement of the inherent variation in outputs between experiments, which will help determine the number of animals required for subsequent experiments. Additionally, the continued development of animal alternatives, such as tissue organoids (mentioned in ‘Replacement’ section above), may lead to the reduction in animal numbers required in

future experiments by being able to address specific scientific questions without the need for whole animals.

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

The published online tools G*Power (Faul et al., 2007 Behavior Research Methods 39(2):175-191) and SISA (http:/www.quantitativeskills.com/sisa/) were used to calculate the approximate experimental group sizes based on existing experimental and published data using these protocols and to determine the minimal number of animals needed for use in each experimental group.

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

Where possible, the number of animals required will be minimised through testing of multiple mutant strains of bacteria (for example, strains deleted for particular genes important for infection) in one experiment, therefore requiring only one control strain on each occasion. Additionally, approaches such as competitive index analysis will be used. This approach tests a 1:1 ratio of mutant and wild type bacterial strains in a co-infection of the same animal (the same overall number of bacteria in the inoculum as per a single strain infection so no cumulative suffering expected). This reduces the number of animals by avoiding the need for control animals as it allows direct comparison of infection and the control in the same animal (by calculating the relative ratio of mutant to wild type in the output).

Tissue and blood from infected animals will also be preserved where possible to generate a biobank of relevant material that can be tested to maximise the output from an experiment without the need for further animals.

Refinement

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

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

The animal models described support the study of host-microbe interactions and replicate several features of the infections associated with the named bacteria in humans, including intestinal infections by EHEC and bloodstream/systemic infections caused by ExPEC. Refinement in this project includes the use of non-lethal models to study bacterial factors involved in host colonisation and persistence.
This allows us to study features of the infection and answer relevant biological questions related to colonisation and transmission whilst reducing animal suffering. Additionally, genomic technologies is helping us identify which key genes are most important during infection. This information will be used to refine animal experiments by potentially offering opportunities to test multiple mutants of bacteria simultaneously within one animal, without

increasing the severity of infection due to the bacterial dose used for infection being consistent. Furthermore, animal stress has been reduced by the adoption of low stress handling techniques.

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

Bacterial infection is a dynamic process that requires the presence of both the pathogen and a mature immune system capable of responding to the pathogen as it grows and adapts to the host environment at the particular site of infection. Adult mice are used in these studies to facilitate an effective response to bacterial infections and limit the risk of an immature animal becoming rapidly overwhelmed with disease. Furthermore, whilst insect/round worm models and more recently zebrafish have been used to study bacterial infection, they offer limited opportunities to understand the adaptative mechanisms of pathogens to body sites and organ structures closely related to humans, as well as the corresponding responses made by host.

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

Procedures and score sheets will undergo continual review by the PPL holder and the local NACWO, NTCO and Veterinary team to ensure any improvements in methodologies that reduce the number animals and degree of severity suffered by animals are employed.

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

Advice on best practice will be followed through alignment with the PREPARE, ARRIVE and LASA guidelines. Additional resources will be obtained through the NC3Rs website and use of their online Experimental Design Assistant (EDA) tool for the design of all new experimental procedures, as well as input on this project from our local AWERB. E-mail updates about 3Rs advances are also regularly provided to researchers at this my home establishment via the mailing lists and intranet sites.

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

The local AWERB, NIO, NACWO, NTCO and Veterinary team regularly inform, and disseminate improvements and recent studies involving reduction, replacement and refinement

Alongside external resources including (but not limited to); collaborators, peers, conferences and lab animal and animal welfare bodies.

During the 1, 3 and 5 year review of the project licence I will update on implementation or consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, score sheets etc. in collaboration with the NACWO, NTCO and Veterinary team with a particular focus on refinements.

27. Establishing a link between bacteria metabolites and colonic cancer

Project duration

5 years 0 months

Project purpose

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

Key words

Escherichia coli, Cancer, Bacteria, Diet, Colibactin

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of the project is to determine if toxin-producing strains of *E. coli* play a role in the development of colonic cancer and whether changes to our diet are increasing the frequency and levels of toxin production by these bacteria. This may help explain why rates of disease are increasing globally.

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

Why is it important to undertake this work?

Colorectal cancer (CRC) is the third most frequent malignant disease worldwide, with 1.85 million new cases and 0.88 million deaths occurring every year. According to current predictions, the global burden of CRC is expected to increase by 20-25%, reaching 2.2

million new cases and 1.1 million deaths per year by 2030. The effect of bacterial metabolites on CRC is under intense investigation, with research showing links between diet, gut microbial community and physiological states of the host. The role of nutrition in the development of CRC has been broadly studied, with research showing positive associations between CRC and the consumption of red and processed meat, inverse correlations between tumour development and fish intake, and the protective effect conferred by the consumption of dietary fibre.

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

Outputs from this work will include greater understanding of the role of a toxin producing E. coli in the development of colonic cancer and whether diet modification could reduce toxin production and limit disease occurrence. Our results will be shared with the scientific community by presentation of the data at conferences and through publication in scientific journals. The information will also be discussed with the general public through events such as Pint of Science, Cafe Scientifique and Science festivals. If the data suggests a strong link between these toxin producing strains and colonic disease, this will be discussed with companies that currently include these bacteria in foods that are marketed as offering probiotic benefits.

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

Short-term, the impact of this research will depend on establishing a link between colonisation of these toxin producing strains and colonic cancer. If this is proven, the longer-term impacts are significant as this may indicate that patients carrying these strains could be more vulnerable to disease. In this case, understanding how and when the toxin is produced and whether diet can influence toxin production will be essential. In the very long term, this may highlight how diets could be changed to reduce colonic cancer development.

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

There is an opportunity to discuss our results with food manufacturers, especially those promoting the use of foods containing live bacteria for health benefits. Through collaborating with such companies, it may be possible to remove or replace strains expressing this toxin. It may also be possible to work with the healthcare sector to determine whether there is a link between carriage of these organisms and cancer development, and this may allow us to consider how diets in vulnerable individuals may be modified to reduce the risk of disease.

Species and numbers of animals expected to be used

- Mice: 240

Predicted harms

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

Adult mice will be used in these studies as they allow us to study the capacity of these toxin producing *E. coli* strains to initiate tumour growth directly in the gut, which is a

complex environmental niche that is difficult to replicate in a test tube. For example, growth of these bacteria in the gut will be affected by factors including availability of oxygen, food, competition from resident bacteria and the need to avoid destruction by the host immune response.

Mice are the model of choice as there is an increasing body of knowledge about how different bacteria within the gut control the growth of bacteria including *E. coli.* There is significant knowledge that allows us to study, in parallel, the animals' natural response to *E. coli* infection.

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

Typically, animals will be given an injection to increase their susceptibility to tumour development, followed by oral infection with bacteria. Animals will further be treated with a drug that modifies the mucosal layer that covers the gut, allowing the bacteria to interact with the cells of the gut wall. Animals will be killed several days after this treatment and any tumour development will be analysed for gross (number and size of the tumours) and microscopic (for cellular dysplasia) changes.

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

Animals are expected to develop tumours in the colon and may consequently experience pain, weight loss and changes to bowel movement as a consequence. In published literature, signs associated with tumours development are observed over a 10-day 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 animal type)?

Up to 90% of animals will be expected to develop tumours as a consequence of these procedures. However, several signs of tumour development are known and will be used to minimise suffering of animals to a moderate or mild level.

Control animals, which will be treated with the drug to increase susceptibility to tumour development but not infected with *E. coli,* may also develop tumours, although these would be reduced in number, with the percentage of animals expected to develop tumours also reduced to around 50%.

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 believe that certain conditions within the gut can cause the *E. coli* to produce the

cancer-causing toxin, colibactin. While it is possible to investigate how the bacteria and its toxin cause induce changes in cells grown in the laboratory, it is not possible to understand the influence of toxin production within the unique environment, found within the gut. As this niche is influenced by numerous other bacteria that grow, survive and modify the conditions found within the colon, it is not possible to replicate these conditions in vitro. Using animals, we hope to understand how diet, modified by the resident microbiome, can influence toxin production and ultimately cancer development.

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

Multiple studies have already been completed in the laboratory using cultured cells. These experiments confirmed that *E. coli* expressing these toxins caused cell enlargement and DNA alterations that are typical of cancerous cells. These changes were not observed when cells were infected with bacteria that did not carry the genes for the toxin, nor when the growth medium used to feed the cells contained specific amino acids that have previously been shown to reduce expression of the toxin.

Why were they not suitable?

Studies carried out on cultured cells provide clear evidence of the ability of these colibactin toxin- producing *E. coli* to cause cellular changes found in cancer. However, they can only provide limited information as they cannot reproduce the the complex and dynamic environment of the gut, which is altered by the bacteria that live there. Many of these bacteria cannot be grown in the laboratory, as they are highly sensitive to oxygen and are often interdependent on each other, making it impossible for them to be grown and studied in isolation.

Reduction

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

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

Published studies show significant differences in the number of tumours that develop in animals infected with toxin-producing bacteria compared to tumour susceptible but uninfected controls. Estimates of the numbers of animals to be used are based on these data. This will allow us to determine whether altering the diet of animals can reduce the amount of toxin produced, reducing tumour development.

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

Animal numbers are based on approximate estimates of usage over a 5-year period. The NC3R Experimental Design Assistant has been used to assist in experimental design.

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

Pilot studies, using small numbers of animals will be used to establish the extent of biological variation in tumour formation between susceptible uninfected and infected

individual animals. Whenever possible, numbers of control animals will be minimised through the testing of several experimental variables (bacterial strains etc) in one experiment. Tissue and blood from infected animals will be preserved where possible to generate a biobank of material, offering an opportunity for further testing without the need to use further animals.

Refinement

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

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

The mouse model described has been developed to understand the role of bacterial infection in colonic tumour development in man. Consequently, tumour development is an inevitable outcome of this study. However, suffering and distress will be limited by careful monitoring of animals, which will ensure that animals will be killed with minimal suffering, while still allowing scientific aims to be achieved.

Data will be continually reviewed to reduce experimental times to further minimise suffering. Animal stress has also been reduced by the adoption of non-aversive handling techniques.

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

Tumour development in this context is believed to be dependent on the capacity of the bacteria to make the toxin and on how the toxin-producing bacteria are controlled by the behaviour of other members of the bacterial gut community. Younger animals fail to control the growth of the *E. coli* as effectively as mature mice and can be rapidly overwhelmed. Terminally anaesthetised animals also offer limited opportunity to study the role of bacteria in cancer development as the development of tumours will occur, over a number of weeks, rather than hours.

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

Procedures will undergo continual review to ensure any improvements in methodologies that reduce the number and degree of severity suffered by animals.

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

Guidance has been sought through the NC3Rs website and use of their online Experimental Design Assistant, and through guidance highlighted by the local AWERB team. E-mail updates about 3Rs advances are also regularly provided.

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

advances effectively, during the project?

Advances in 3Rs are continually provided including email updates, 3Rs seminars and culture of care subgroup outputs. These provide updates on new approaches and provide guidance regarding published methods. There are also opportunities to discuss new methods with the Named Veterinary Surgeon (NVS) and the Named Training and Competency Officer (NTCO) who offer help and support around training for the adoption of new methods. The NC3R website provides an additional excellent resource.

28. Developing novel neuromodulation strategies for the treatment of brain disorders

Project duration

5 years 0 months

Project purpose

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

Key words

brain disorders, brain stimulation, brain-computer interface, neurology, psychiatry

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is understand how specific parts of the brain control movement and cognition and to use this knowledge to develop new treatments for brain disorders using electrical stimulation. We will use ways of monitoring and manipulating the brain, including optical stimulation, to identify new targets and patterns for electrical stimulation, and test the capacity of these novel approaches to change brain activity and behaviour in ways that could provide new or better therapies for brain 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?

Brain disorders are common and cause considerable suffering and cost the UK economy more than £100 billion per year. Drug-based treatments for brain disorders have stalled and many pharmaceutical companies have reduced their investment in these diseases, but treatments are urgently needed. Stimulating the parts of the brain with electrical current provides an alternative way of treating brain disorders, and has proven an effective treatment for disrupted movement in Parkinson's Disease. Finding ways of using brain stimulation more effectively and for other brain disorders could alleviate the suffering of people with these diseases and provide significant economic benefits.

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

We aim to produce the following outputs:

Publications describing novel findings as to how specific brain areas control and contribute to movement and cognition

Publications describing novel methods of stimulating the brain and the effects of these methods on brain activity and behavior

Publications and/or patents describing specific code and/or electrical circuits to deliver novel methods of brain stimulation

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

Short/medium term

The sizeable community of researchers working on function/dysfunction in the basal ganglia and their partner brain circuits, including those researching the causes and consequences of neurological disorders.

A wider community of researchers in neuroscience, neurology, psychology and bioengineering. Bioengineers who can use the circuits and computer code to design new medical devices.

Long term

Patients with brain disorders will benefit from findings that ultimately influence clinical devices.

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

Publish our data on "preprint" servers and in peer-reviewed international journals.

Share our data (published and unpublished) through presentation at conferences as well as at specialist user groups (e.g. clinicians, industry). We will share raw data on sharing repositories.

Communicate and share are findings on other platforms such as the MRC Brain Network Dynamics Unit (MRC BNDU) is committed to supporting a culture of open science, including the timely sharing of well annotated primary data sets (and metadata) that are generated by our research using animals.

Through public engagement activities, such as school and patient group open days.

Species and numbers of animals expected to be used

- Rats: 5250
- Mice: 17500

Predicted harms

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

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

In our project, we will mostly use adult small rodents (mice and rats) for the following reasons:

They are the most widely used animals that have sophisticated motor and cognitive behaviours. The anatomy and physiology of the mouse and rat brain is well described. The experimental methods that will be used have been specifically designed for and successfully used in this species.

In rare cases we will use juvenile animals to look at the connectivity between brain areas measured in brain slices.

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

Some animals will be anesthetised and have surgery to place or implant devices in their brains, so that we can monitor and manipulate animals' nerve cells and behaviour. These experiments typically take place over weeks or months.

We will motivate the behaviour of some animals by carefully controlling their access to food or fluids. We will then deliver food or fluids in response to specific behaviours. These experiments typically take place over weeks or months.

Some animals will have devices for recording and stimulation of brain activity permanently attached to their heads.

Some animals will have their heads temporarily fixed in place while they are awake, so that we can monitor and manipulate animals' nerve cells and behaviour. These experiments typically take place over days or weeks.

Some animals have substances injected into their brains to mark nerve cells and the connections between those nerve cells that can under a microscope after the animals brain has been removed (post mortem). These experiments typically take place over weeks.

Animals will be killed by a humane method and, typically, tissues taken for analysis after death.

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

Animals that have had surgery will experience some pain and discomfort, but are expected to recover quickly (over hours) and will be given painkillers and post-operative care, just like people recovering in hospital.

Animals that have the availability of food or water controlled will be hungry or thirsty for short periods (over hours), but we do not expect this to impact greatly on their wellbeing.

Head-fixed animals might initially experience frustration or stress for short periods (over minutes), but we do not expect this to impact greatly on their wellbeing.

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

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

Rats:

90% Moderate

10% Mild/Subthreshold

Mice

30% Moderate

70% Mild/Subthreshold

(Mice have a large percentage of mild due to their greater numbers on breeding protocols)

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

- Killed

Replacement

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

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

Studies in animals provide the best way of studying cognitive and motor aspects of behaviour and the brain processes that control them. Many brain diseases or the symptoms of those diseases can be accurately modeled in rodents. Recording and stimulating brain activity in animals greatly facilitates the development of novel treatments for brain disorders.

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

Computational modelling of the brain areas of interest

Why were they not suitable?

Our knowledge of the mammalian brain and the complexity of computer simulations is not advanced enough to replace the experiments in that can address our scientific aims,

Reduction

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

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

We have estimated the number of animals based on our experience of the number of experiments we plan to perform to address our scientific objectives and that we have performed in similar projects.

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

Where ever possible we apply experimental designs that compare parameters within animals. For example, we can use the same animal to test the effect of different patterns of brain stimulation on the activity of nerve cells and behaviour. This allows as to evaluate the importance of the brains temporal dynamics very precisely in the same animal.

For experiments involving behaviour and/or the effect of brain stimulation on neuronal activity. we identify statistical methods and experimental variables that will allow us to control for as many variables as possible using limit numbers/groups of animals.

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

We will use both male and female animals in the majority of experiments and use efficient breeding strategies.

We will make use of pilot experiments to provide proof-of-principle that new experiments are viable before committing to enough animals for statistical analysis.

We have designed experiments that maximise the amount of behaviour and/or recordings of brain activity that we can obtain from individual animal. This produces data of high quality and quantity that reduces the overall number of animals that need to be recorded. We make extensive use of in silco simulations to develop methods of brain stimulation that deliver stimulation in response to detecting specific aspects of brain activity.

Refinement

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

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

Disease models. We will use methods that model aspects of brain disorders, while still allowing animals to behave normally with respect to feeding, grooming and other normal behaviours. For example, by destroying a group of neurons on one side of the brain only, animals have unaffected function on one side of the body and/or have mild cognitive impairment that does not impair their ability to perform these normal activities.

Implanting devices to record and stimulate brain activity. Many animals will undergo cranial surgery to implant devices that allow the recording of brain activity over weeks or months. While surgery will cause initial pain and discomfort over hours or days, animals receive medication to reduce this and, once recovered, are able to behave normally without further distress or discomfort.

Recording and stimulating brain activity. We use methods of placing electrodes into the brain that are aimed at reducing disruption to normal behaviors by being as small and light as possible, while still enabling many brain areas to be monitored simultaneously.

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

Using animals that are less sentient than rodents would not provide and adequate model to study neural activity and behaviour that is relevant to function of motor control and cognition in humans and the treatment of human brain diseases. The brain areas that we study are similar enough to humans to make a valid comparison.

The majority of experiments will require the fixation of a device to the animals skull. It is necessary to use adult animals for all of these experiments, as larger animals can support such devices without disrupting normal behaviour

While some questions can be addressed using terminal anaesthesia, in most cases the brain activity associated with either function or dysfunction in the brain areas of interest cannot be reproduced accurately unless the animal is awake.

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

Operative and post-operative pain relief. All animals that undergo surgical procedures receive pain relieving medication that lasts for several that lasts for several hours after the procedure and can receive further medication until recovery is complete

Post-operative care. We have introduced high energy palatable foods that prevent post-operative wieght loss and will look for other methods to speed post-operative recovery.

Video monitoring. Many of our recording set ups have video that can be used for enhanced monitoring of animals.

Cranial Implants. We will continue to try and achieve smaller and lighter implants for head-fixation and recording/stimulation of neuronal activity. We will monitor alternative materials and/or designs that could achieve this.

Wireless recording. For recordings in freely-moving animals, we will explore the possibility of implanting self contained recording/stimulating devices that can operate wirelessly. This would provide less restriction on the animals behavior.

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

We receive all updates for NC3Rs – National Centre for the Replacement, Refinement and Reduction of Animals in Research. https://nc3rs.org.uk These include webinars, links to specific relevant publications e.g. Minimising mouse aggression, Sci reports (Oct 2019) as well as principle and practice videos. Experiments will be set-up in line with the PREPARE guidelines. We will follow LASA guidance on aseptic surgery and administration of substances. All of our publications will comply with the ARRIVE guidelines.

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

As part of the host institutions structure for regulating animal work all licence holders must attend termly animal welfare meetings. These groupings are organised to map onto local AWERBs, ensuring that participants share common research practices. A specific agenda item for these meetings explores advances in the 3Rs, ideas are exchanged and best practice discussed. Three crucial communities also present at these meetings are the vets, NACWOS and the Regional Programme Manager NC3Rs – National Centre for the Replacement, Refinement and Reduction of Animals in Research, all of whom provide updates and advice for implementation of any advance.

29. Investigation of Rift Valley fever virus (RVFV) invasion of the brain using an intranasal infection model in the ferret (Mustela furo)

Project duration

2 years 0 months

Project purpose

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

Key words

Zoonosis, Encephalitis, Virus, Intranasal

Animal types	Life stages
Ferrets	juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to develop an intranasal infection model in ferrets to investigate Rift Valley fever virus infection and cell tropism in the brain in humans.

A further aim will be to share biological material produced by this study with collaborating partners in Europe.

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

Why is it important to undertake this work?

Rift Valley fever virus is a significant zoonotic disease in Africa and parts of the Middle East. It causes devastating outbreaks in human and livestock populations with a range of severe disease manifestations including febrile illness, haemorrhagic fever and

encephalitis. Brain infection (encephalitis) as a result of infection with Rift Valley fever virus is rare but can have damaging consequences of infection for humans who experience it. Globally, Rift Valley fever virus is poorly studied as rodent models do not consistently replicate infection of neuronal tissue. Consequently, results are not robust enough to be generalised to wider animal or human infections. The ferret offers an alternative model that reflects many features of human physiology and pathology, which make it suitable for understanding Rift Valley fever virus infection in humans.

The study is funded by the European Union to encourage sharing of reagents and research samples from investigations. This study will generate biological material that can be shared with researchers in Europe to increase the information generated by this project and avoid repetition of this experimental study by other investigators. This paradigm reduces the requirement for future animal studies in this area and minimises the amount of biological material wasted following sampling as multiple assays will be undertaken.

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

Development of a novel well characterized model of RVFV encephalitis that can be extended to non- ferret mammals.

Improved detection of RVFV in brain tissue samples and the ability to visualise virus infection in the context of brain structures and any associated neuropathology.

Biological products, such as whole genome assessed cultured virus and formalin fixed tissue samples, for the scientific community

Collaborative publications on RVFV infection using ferrets as a model.

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

Researchers investigating viral encephalitis in models of human disease. This benefit will be apparent upon publication of data, within three years following the start of the project.

European researchers working in the field of virus infection and Rift Valley fever detection. This will be a short-term benefit as collaborative partners receive biological samples from the initial investigation.

Medical pathologists who want to differentiate RVFV infection in humans. This benefit will be apparent upon publication of data, within three years following the start of the project.

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

The findings of this study will be published in a project report to the European Community (via the VetBioNet Project) and in a peer-reviewed journal. Due to the multidisciplinary nature of the project it is likely that more than one publication will arise as the investigation will look at a multifaceted approach to understanding RVFV infection in ferrets. This paradigm maximises research outputs and therefore wider scientific community understanding, while minimising the animals required for such detailed outputs.
Shared knowledge with European collaborators in understanding RVFV infection in a ferret model. Following publication of findings from this investigation we would also disseminate this knowledge at national and international conferences in the hope that it maximises its potential target audience and can assist in understanding RVFV impact globally.

Samples collected will be made available through the VetBionet transnational access program to enhance scientific collaboration.

Species and numbers of animals expected to be used

- Ferrets: 18

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 ferret has been successfully used as a model for intranasal infection and aerosol transmission for viruses such as Influenza A and SARS Coronavirus 2. It has also been shown to be susceptible to RVFV infection (Barbeau et al., 2020, mSphere 5, e00798). Consequently, ferrets are likely to make an ideal model for understanding intranasal exposure to RVFV, and multiple samples can be generated from a single animal, which minimises the need for high animal numbers and increases potential research outputs. In this study we plan to use this approach to understand the mechanism of brain infection following intranasal exposure to virus preparations to elucidate this transmission route and its subsequent impact. This data will provide a deeper understanding of RVFV in humans as the ferret model has similar physiology and pathology.

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

Animals will undergo short-term anaesthesia prior to any procedure. Individual ferrets will have a biochip inserted subcutaneously to facilitate individual identification and allow remote measurement of body temperature. A non-surgical removal of blood (pre bleed; <2mL) from a superficial blood vessel will take place before the infection study begins. One nasal wash, oral and rectal swab prior to and one after virus challenge. Virus challenge, which consists of an intranasal inoculation with a defined quantity of RVFV, which is known to infect ferrets (or tissue culture medium for control animals).

Animals will be randomly assorted into groups of three (experimental n=15 animals, control n=3 animals) and sampled at defined intervals or until animals develop signs of virus infection of the brain, at which point they will be killed by a Schedule 1 method to prevent unnecessary suffering.

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

As infection of the brain occurs, infected animals may experience a raised body temperature, early neurological signs and agitation. A quantitative clinical assessment in association with a decision algorithm will be used define a humane endpoint for the experiment. Given the intranasal route of virus instillation, the animals could develop upper or lower respiratory tract infection presented as sneezing or coughing. Due to daily, routine observations the duration of these effects would be detected within 12 hours.

If disease progresses rapidly, the animals could present with reduced activity and limb weakness as the virus infects further regions of the brain. This could be associated with

increasing pain, weight loss (due to inappetence) and aggression. It is likely that if allowed to progress some animals would die from the infection within a number of days.

With daily observations and welfare assessments we will ensure that these effects do not occur. At first sign of any of these signs we would immediately treat this as a humane endpoint to the investigation and terminate using schedule one technique. A detailed clinical scoresheet will determine the earliest possible endpoint.

There may be adverse responses to anaesthesia, as has been observed occasionally in other ferret studies, and all animals would be monitored until they are assessed as being fully recovered and active. Animals may experience mild pain from the pre-bleed puncture wound.

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

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

Animals infected with RVFV would develop encephalitis and experience severe pain. We will identify early signs of infection (raised temperature / behavioural changes) and terminate the experiment when animals reach moderate pain levels as indicated on the clinical scoresheet. It would be expected that 80% of animals in the virus challenge group would reach this severity.

Control animals would not be expected to develop any adverse effects following inoculation with tissue culture medium. However, these animals could develop short-term sneezing and coughing following intranasal exposure of buffer solutions.

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 biological process of neuroinvasion by a virus, which involves crossing the olfactory portion of the nasal cavity and into the brain via the bony ethmoidal tissues, cannot be recreated in cell or tissue culture. Visualising the infection process in the context of a complex organ structure such as the brain requires a living animal. These outputs are essential for understanding exposure to RVFV-containing fluid and subsequent impact of RVFV in mammals.

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

A neurological cell model of infection, potentially developed in a three-dimensional matrix might offer the ability to investigate the cellular response to RVFV infection.

Why were they not suitable?

Cell culture or organ models cannot show structures that are found in the intact brain or respiratory tissues that are present within the nasal cavity, and therefore no inference on pathology and viral tropism following infection can be made.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 planned to use 15 animals within the virus challenge group and 3 age-controlled animals as un-infected controls. The 15 animals infected with virus will be randomly allotted into 5 groups and sampled at 5 pre-defined timepoints to day 21 post inoculation.

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

The numbers estimated for use are the minimum number required to deliver sufficient infected animals that will be sampled at three day intervals with five sampling timepoints. Groups of three will generate sufficient infected material to investigate disease outcomes and offer the ability to share biological material with European collaborators (the ultimate goal of the VetBioNet project). An assessment of the study design by the organisations statistician with the study of Barbeau et al., 2020, concluded that the use of multiple timepoints should provide more power to compare viral load between tissues as well as between stages of disease development.

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

This project will maximise the volumes of blood and tissue samples derived from each animal to enable sharing of biological material derived from this experiment. Samples will include serum, fixed organ tissue and extraction of RNA from fresh tissue. We will avoid duplication of this type of study and thus reduce the number of animals needed to achieve scientific goals.

Refinement

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

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

The intranasal inoculation method will be used to instil virus into the nasal cavity. This is the most effective way to enable infection of virus through the olfactory bulb to simulate exposure to virus- containing fluid and reduce the need for inoculation by injection, which would increase the potential harm caused to experimental animals.

Ferrets will be co-housed in their groups as they are naturally a social animal and provided environmental enrichment to encourage normal behaviour.

The anaesthesia protocol has been significantly refined in recent projects using ferrets as a model for Influenza A infection to make it shorter and safer to use without adverse effects with inhalational and reversible agents.

A clinical score table have been developed for this project that considers a range of potential disease signs related to particular organ systems that may be affected by the protocol. It will be used to identify endpoints. These will be reviewed at the end of the project to identify any improvements that could be introduced.

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

Due to a similar physiology and pathology the ferret is the only mammalian model available that will experience neuroinvasion following RVFV aerosol inoculation in a similar manner to infection in humans. In addition, non-vertebrate animals would not facilitate meaningful insights into RVFV in mammals.

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

Ferrets will be kept for a period of acclimatisation, co-housed in cages and provided environmental enrichment.

A clinical scoring system will be used to monitor all animals participating in the study for early identification of clinical signs or signs of distress. Where necessary, increased monitoring of animals will help to reduce harm to individuals.

Ferrets undergoing anaesthesia will be provided with extra cover to ensure they remain warm during the procedure.

A NVS with extensive experience in using ferrets will be available throughout the duration of the project.

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

We will follow the guidance on housing and husbandry of ferrets as per the Code of Practice and review recent literature that has used ferrets as a model to ensure we are operating the most refined, up-to-date methodology and best practices.

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

The project licence holder will check the 3Rs website (https://www.nc3rs.org.uk/) and other accessible sources of information on animal challenge models. This includes information on the housing and husbandry of ferrets (https://nc3rs.org.uk/housing-and-husbandry-

ferrets), legal guidance on housing ferrets (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388 535/CoPanimalsWeb.pdf) and lists appropriate conditions for their maintenance.

The project license holder will ensure that all researchers are kept up to date with current guidelines and practices by having direct conversations before, during and after the experimental protocol, and recording evidence of these welfare meetings. The NIO will provide up to date guidance on welfare and housing of ferrets for the duration of the project.

30. Developing vaccines against infectious diseases and 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

Key words

vaccines, immunology, antibodies, T cells

Animal types	Life stages
Mice	adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to test new candidate vaccines and therapeutics in a small animal model (mice), enabling us to evaluate their effectiveness and safety, to optimise their design, and to select the most promising candidates for assessment in human clinical trials.

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

Why is it important to undertake this work?

Vaccines are among the greatest success stories of modern medicine. Even prior to the covid-19 pandemic, the World Health Organisation have estimated that vaccines prevented at least 10 million deaths worldwide between 2010-2015. However, the emergence of covid-19 and its devastating impact on worldwide health and economic prosperity has highlighted the critical importance of developing new vaccine technologies against current and emerging infectious diseases. New vaccine technologies including

mRNA and viral vector vaccines that have not previously been used widely in mass vaccination programs have been at the forefront of mankind's response against covid-19. Pre-clinical studies in small animal models are an essential pre-requisite for development of new vaccines. The immune systems of mice have been extensively characterised, and decades of research has shown that many aspects of the mouse immune system behave in a similar way to the human immune system making the mouse an excellent small animal model for vaccine studies. Almost all of the vaccines and therapeutics successfully used in humans today would not have been developed without studies in mice.

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

Identification of several lead candidate vaccines against infectious agents such as bacteria or viruses that pose a major risk to human health. Accumulation of data on safety and effectiveness to support further development of these products into clinical trials.

Identification of lead candidate cancer vaccines and/or therapeutics; accumulation of data on safety and effectiveness to support further development of these products into clinical trials.

Improved vaccination strategies, and a better understanding of the type of immune responses required to generate protective immunity against target infectious organisms and for the successful treatment of cancers.

Improvements in our understanding of new vaccine technologies, increasing their versatility, and exploring new ways in which we can use these technologies to develop better vaccines.

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

In the short term, promising data from pre-clinical studies in mice will support translation of our lead vaccine candidates into clinical trials in humans. Data generated from pre-clinical studies will be publicly disseminated where appropriate, in scientific publications, and at international conferences enabling others in the field to benefit from the knowledge gained from these studies. Collaborations with other scientists may be initiated to explore new ways to utilise new vaccine technologies.

In the medium to long term, new efficacious vaccines against difficult to target infectious diseases, and new treatments for cancer would considerably improve the health and wellbeing of the general public, both in the UK and worldwide. As the recent covid-19 pandemic has demonstrated, improvements in pandemic preparedness strategies could also have significant economic benefits to society in future pandemic scenarios. Improvements in our understanding of novel vaccine technologies could have wider implications for scientific research, leading to further research and innovation in new areas of biotechnology.

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

The aim of this project is to develop new effective vaccines. To achieve this aim, pre-clinical studies in mice will be essential to support further testing of lead candidate vaccines in human clinical trials. We are already conducting human clinical trials with two candidate vaccines, and over the next five years we will design new vaccines and therapeutics to target other infectious diseases and certain types of cancer by generating an immune response against them. We have already formed, and will continue to form

collaborations and agreements with other scientists to secure the expertise and facilities required to accelerate development of our lead candidate vaccines. Data from pre-clinical studies will be disseminated where appropriate through publication in scientific publications, and will be presented at national and international scientific conferences.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

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

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

Adult mice (typically from 6-8 weeks of age) will be used for this project. Mice are the animal species that have the best characterised immune system. Mice have been used in the vaccine field for decades as a model species in which to test new candidate vaccines, and immune responses generated by vaccines in mice have generally proved to be very good indicators of vaccine effectiveness in higher species including humans. Almost all currently available vaccines and therapeutics have at some stage been tested successfully in a mouse model. Because the mouse immune system has been so well studied, numerous tools are available to study vaccine induced immune responses and mechanisms of protective immunity that are not available in higher organisms. We typically use adult mice, since these animals have a mature immune system and therefore generate stronger immune responses making it easier to test new vaccines.

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

In a typical experiment, mice would first be injected with a candidate vaccine. This would typically be performed intramuscularly (i.e. injection into the muscle, which is how vaccines are normally administered in humans) but may also occur by other common vaccination routes including intranasal or oral. Intramuscular injections are performed with the mice under general anaesthetic to reduce pain and to restrain animals. After a period of time, typically between two and three weeks, a sample of blood (<10% total blood volume) may be taken from the tail vein to assess immune responses. Following the tail vein bleed, mice could receive a second dose of candidate vaccine as previously described.

Between two and three weeks after the second vaccination the mice will be killed, after which blood and tissues will be collected to assess immune responses.

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

Discomfort as a result of vaccine administration and/or a mild local reaction to the vaccine with symptoms including redness and swelling at the administration site. Pain will be significantly reduced using anaesthetic, and should be transient with the animal recovering rapidly without lasting impairment. Discomfort may increase upon repeat administrations, but the number of repeat administrations and the time interval between repeat administrations will be restricted.

Systemic reaction after vaccination due to overstimulation of the immune system. A human would experience a systemic reaction as fever, fatigue, headache etc but mice can present with piloerection (ruffled fur), altered behaviour and in some cases changes to breathing pattern. We would expect these symptoms to be rare and to improve rapidly (within 24 hours).

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

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

Mice - 90 % mild, 10% moderate

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

- Killed

Replacement

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

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

It is essential for the development of any candidate vaccine to demonstrate that the vaccine can generate a strong immune response, because it is through immunity that vaccines can provide protection from a target infectious disease. The mammalian immune system is incredibly complex. The generation of an immune response involves the interaction of a large number of different cell types, in multiple different organs located at different sites throughout the body. It is therefore impossible to adequately replicate a mammalian immune system in tissue culture, in silico, or in lower organisms such as bacteria or yeast. The mouse immune system has been extremely well studied, and has been used extensively in the testing of vaccines and therapeutics. We can therefore use the mouse model to effectively evaluate the potential efficacy of early vaccine candidates without needing to test the candidates in more sentient mammalian species.

Which non-animal alternatives did you consider for use in this project?
It is not possible to adequately replicate a mammalian immune system using any existing in vitro, ex vivo or in silico techniques or models. However, where possible we will replace studies in mice with ex vivo or in vitro techniques. For example, a critical aspect of vaccine research is to assess whether antibodies generated by a vaccine can prevent infection by a target disease organism. Using serum from vaccinated mice, we can perform an in vitro neutralisation assay to test this, rather than experimentally infecting the animal with an infectious organism. In vitro neutralisation assays have been shown to strongly correlate with protective efficacy in both mice and humans for many disease targets, and so we are confident that we can effectively replace animals in this aspect of our research without compromising in scientific rigor. We will also ensure that we perform extensive quality control testing and screening of vaccine candidates in vitro (such as testing that they have the required physical and chemical characteristics) before administering them to animals (thereby replacing animals in the early screening process).

Why were they not suitable?

Since the mammalian immune system is highly complex, involving multiple different cell types within multiple different organs, it is impossible to replicate the complex interaction between these cells and organs without studying them in a complete, living, mammalian organism. This is why ex vivo culture of immune cells and in vitro 'organ on a chip' technology cannot recapitulate the generation of an immune response in a mammalian species after vaccination. Indeed, our current understanding of the mammalian immune system is also insufficient for us to be able to adequately model the interaction between vaccines and the immune system in silico using computer simulations.

Reduction

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

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

Experience from previous vaccine studies has been used to assess the number of experiments in animals likely to be conducted over the next five years. In each animal experiment, the number of groups required will be determined by the number of different vaccines we need to test, and the number of control groups required. The number of groups per experiment, the number of mice per group, and the total number of experiments required to select each lead vaccine candidate have been estimated based on our previous experience of performing pre-clinical studies in mice prior to initiation of clinical trials in humans.

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

We have consulted current PREPARE guidelines on the experimental design of animal studies, to ensure that studies are as scientifically rigorous, reproducible and translatable as possible, thereby reducing the need to repeat experiments. Studies in animals will be performed with a clear hypothesis and formulated through extensive analysis of recent literature and our own experience to ensure that all experiments are informative and necessary. Group sizes (typically n=6-8) were designed to be as small as possible but sufficient for firm (statistically significant) conclusions to be drawn. Power calculations were used to determine these optimum group sizes. Mice will be obtained through commercial suppliers, and only the exact number required for each experiment will be ordered. They will also be age and sex matched, to increase the reliability of the data. Where possible, data obtained from each animal will be maximised through the collection of multiple tissue samples and/or sequential sampling from the same animal i.e. collection of blood samples from the same mouse at multiple timepoints rather than using several mice. On publication of our data generated using animal research, we will endeavour to comply with current ARRIVE guidelines on the conduct and reporting of animal experiments.

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

Early candidate vaccines will be quality control tested and screened in vitro prior to starting animal studies to reduce the number of vaccines that require testing in animals and ensure than only high quality prototype vaccines are used. We will perform pilot studies using small numbers of animals to test new concepts prior to conducting larger studies that require more animals. As we proceed with our product development program we will introduce project GO/NO-GO points to assess progress and to enable us to decide in an efficient manner whether or not to continue with a particular vaccine project, thereby avoiding the unnecessary use of mice in projects that will cease to continue.

Refinement

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

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

This project will require the use of adult mice to test the effectiveness of new candidate vaccines and therapeutics. The project will involve the administration of substances (such as vaccines and therapeutics), primarily through injection into the muscle (since this is the route of administration most commonly used in humans). Intramuscular injection will be performed under general anaesthesia to reduce pain and stress. Best practice will always be followed regarding administration volumes and frequency. Blood samples may be taken during the course of each experiment to assess immune responses. Best practice will be followed regarding the volume and frequency of blood sampling, as well as sampling technique. Animals will be humanely killed at the end of the experiment.

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

Vaccine induced immune responses in the mammalian immune system are highly complex physiological processes and cannot be adequately modelled in lower organisms, necessitating the use of a mammalian species. The mouse immune system has been extensively studied and has been shown to serve as a highly translatable model system for vaccinology, enabling us to avoid using a more sentient mammalian species. Adult mice are required for these studies, since they have a mature immune system and therefore generate stronger immune responses.

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

Substances will be administered at the lowest doses possible and with the fewest number of administrations possible to achieve the required effect, reducing the likelihood and severity of pain and reactogenicity. The number and frequency of repeat administrations will be restricted and kept to a minimum. Quality controls will be performed on candidate vaccines and therapeutics, including endotoxin testing of recombinant proteins, to reduce the risk of adverse events. As our primary aim is translation into human clinical trials, we will mainly use adjuvants (substances designed to increase immune responses) that are approved for use in humans (or substances that are bioequivalents of approved

adjuvants). We will also avoid using adjuvants that are known to be ulcerative, such as Freund's adjuvant. As stated previously, intramuscular injections will be performed under general anaesthetic to reduce pain, with the depth of anaesthesia controlled to avoid unnecessarily deep or prolonged exposure. The frequency of blood sampling will be kept to a minimum. From previous experience we can identify the most informative sampling timepoints for measuring different types of immune response, avoiding any unnecessary sampling.

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

In order to ensure compliance on 3Rs guidance, publications by the National Centre for the Replacement, Refinement and Reduction of Animals in Research will be followed. The norecopa website (https://norecopa.no) also contains a variety of links to current guidance on best practice in conducting animal experiments, such as the PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence). The Laboratory Animal Science Association (LASA) also publishes guidance on best practice (https://www.lasa.co.uk). Our establishment's Named Information Officer can provide further guidance.

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

We will regularly check for updates to 3Rs guidance on the website of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (www.nc3rs.org.uk) and the RSPCA website on guidance for the use of animals in research (https://science.rspca.org.uk). We will attend regular internal 3Rs meetings at the establishment, and seek guidance from the NC3R's regional manager.

31. Studies of cancer inflammation and immunity in vivo

Project duration

5 years 0 months

Project purpose

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

Key words

Immunotherapy, Inflammation, Tumour immunity, Immune-escape, Cancer immunology

Animal types	Life stages
Mice	adult, aged

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Our main objective is to elucidate which signals trigger anti-tumour immune responses, and to distinguish mediators favouring tumour elimination from those that support cancer progression.

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

Why is it important to undertake this work?

The recently reported unprecedented efficacy of immunotherapy in a proportion of cancer patients has revolutionised the way we treat cancer. In turn, this has highlighted the need for more mechanistic studies to determine why some patients show partial response or do not respond. In this context, the study of the mediators that regulate the function of immune cells in the vicinity of the tumour, distinguishing mediators promoting tumour immunity from those that support tumour growth, is critical to our ability to maximise the efficiency of therapy for cancer patients. The benefits of this project will be: 1) The identification of drivers of natural and therapy-induced tumour immunity in mouse models of

cancer that recapitulate the human disease. 2) To inform and advance our ability to identify cancer patients likely to respond and benefit from cancer therapies aimed at harnessing the anti-tumour activities of the immune system. 3) The generation of novel therapies to disrupt immune suppression and enhance the efficacy of conventional and immune-based cancer treatment in patients.

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

The output of the research will be shared with other researchers in the field and the broader community through presentations in conferences (nationally and internationally) and publications in peer-reviewed journals. In addition, our work has direct translational and clinical applications that we will investigate through collaborations with clinicians at the Establishment and other medical cancer centres worldwide.

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

The benefits are:

The identification of regulatory pathways of natural and therapy-induced tumour immunity using mouse models of cancer that recapitulate the human disease; distinguishing inflammatory mediators that favour tumour elimination from those that support cancer progression alterations in preclinical models (short-term).

To inform and advance our ability to stratify subgroups of cancer patients with an immune-promoting tumour environment who are likely to benefit from existing immunotherapy from those with inhibitory pathways resulting in local immune suppression (mid-term).

To further inform the design and guide patient stratifications in investigator-led clinical trials that will be ran in multiple centres across the UK in which the scientific rational was provided by work undertaken in our previous PPL (mid-term). 4) To develop novel targeted interventions to disrupt immune suppression, stimulate the tumour inhibiting function of the immune system and enhance the efficacy of conventional and immune-based cancer treatment in patients (mid and long-term).

We will achieve these benefits through the use of: 1 genetically engineered mouse lines with select immune and inflammatory alterations, 2 genetically engineered mouse models of cancer, and 3 the use of cutting-edge immune profiling techniques in both animal models and patient samples.

By combining fundamental and translational research our ultimate goal is to develop novel targeted interventions to enhance the efficacy of cancer treatments that harness the anti-tumour function of the human immune system.

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

Our findings will be made available to other scientists through collaborations, publication in high profile peer-reviewed journals and presentations at scientific conferences and meetings. Our Institution has a policy of ensuring that all publications generated are available on free access to all.

Species and numbers of animals expected to be used

- Mice: 9200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 development and function of an inflammatory and immune response involves many different cell types interacting in a dynamic three-dimensional environment. For example, the progression of an immune response within a whole organism involves changes of antigen expression and presentation that evolve with both time and spatial distribution. Similarly, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding cells, governed by multiple signals originating from both their immediate neighbours and from distant tissues. These factors combined with the involvement of multiple host cell-types and the expansion and migration of rare tumour-specific effector cells mean such research cannot be carried out in tissue culture alone or reproduced in silico and can only be addressed with the use of animals.

The mouse is one of the model organisms that most closely resemble humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are functionally conserved. Further, mice have genes not represented in other animal model organisms (e.g. Caenorhabditis elegans, i.e. nematode worm, and Drosophila melanogaster i.e. fruit fly) such as those involved in adaptive immune responses. Mice can be genetically altered, there is extensive literature concerning the topics of our investigation, and our own studies can be enhanced by combination with many complementary models developed by others in the field. Definitively, mouse models are important for placing the findings of in vitro (test tube) studies or correlative analysis of human samples into an appropriate and meaningful in vivo (living organism) context. It is the combination of in vitro, in silico (computer) and in vivo studies that provides the insight needed to understand cancer biology and develop new therapeutic approaches, and there are no effective approaches to hand that can replace the in vivo studies, as these allow the in vitro findings to be tested in an appropriate environment. For our studies we need animals with a functionally mature immune system, therefore we will only use adult mice.

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

Mice may have tumours induced by chemical agents or implanted subcutaneously under the skin, intravenously, orthotopically (e.g. intradermally for skin cancer cells of in the mammary fat pad for breast cancer). The growth of tumours will be assessed by either imaging with ultrasound or when possible, such as in the case of subcutaneous or intradermal tumours, using callipers. Some mice will be irradiated to replace their immune-system with specific populations of cells from donor mice or to treat their tumours. Chemical agents, such as existing cancer therapies, potential new therapies or agents may be administered by a variety of routes including orally or by injection. Typically, a single mouse will not experience more than 4 procedures.

Some mice will undergo surgery, for primary tumour removal, 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.

The health of all mice will be observed daily. Notwithstanding this, all the mice will be humanely culled at the end point of the experiments.

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

The majority of animals (up to 95%) are not expected to show signs of adverse effects that impact on their general well-being apart from the development of tumours. The vast majority of the procedures will result in no more than transient discomfort and no lasting harm. However, some mice in which we are investigating the effects of treatments upon the spread and progression of tumours (~3% based on recent experience) might be unexpectedly found dead overnight when monitoring indicates that they are normal. This can be due to a sudden impact of the spread of cancer cells from the original location to other body organs. The health of all mice will be observed daily. Notwithstanding this, all the mice will be humanely culled at the end point of the experiments.

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

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

Based on our vast experience using these procedures and experimental models we anticipate about 85 to 90% of mice to experience Moderate severity, 10 to 15% Mild, and less than 1 to 3% a Severe one.
Thus, the vast majority of mice are only expected to experience mildest to moderate clinical symptoms due to tumour growth before they are humanely killed. Some mice will experience the discomfort of repeated (daily) injections of therapeutic agents or oral delivery. We will aim to utilise the least stressful route of administration wherever possible.

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 and function of an inflammatory and immune response involves many different cell types interacting in a dynamic three-dimensional environment. For example, the progression of an immune response within a whole organism involves changes of antigen expression and presentation that evolve with both time and spatial distribution. Similarly, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding cells, governed by multiple signals originating from both their immediate neighbours and from distant tissues. These factors combined with the involvement of multiple host cell-types and the clonal expansion and migration of effector cells mean such research cannot be carried out in tissue culture alone or reproduced in silico and can only be addressed with the use of animals.

The mouse is one of the model organisms that most closely resemble humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are functionally conserved. Definitively, mouse models are important for placing the findings of in vitro studies or correlative analysis of human samples into an appropriate and meaningful in vivo context. It is the combination of in vitro and in vivo studies that provides the insight needed to understand cancer biology and develop new therapeutic approaches, and there are no effective approaches to hand that can replace the in vivo studies, as these allow the in vitro findings to be tested in an appropriate environment.

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

Non-animal alternatives are cancer cell lines, primary immune cells and tumour explants. We use these routinely in our research however the aims of this study cannot be achieved without using living organism. There are not good alternatives to address our research questions and achieve our translational goals. Additionally, use of animals will be minimised by bioinformatic analysing publicly available datasets through computational means and, where suitable, further using in vitro model systems.

Why were they not suitable?

The study of cells in culture (in vitro) and less sensitive organisms 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 to allow us to investigate the impact of the relationship between the immune systems in the promotion of cancer cell growth and spread.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the minimal number of animals.
Based on past experience, group sizes of between 5 and 8 animals (dependent on the readout, typically fewer for transplanted tumours compared to spontaneous tumours in GEMM mice) per experimental group suffice. For instance, in implantation experiments where we deplete a gene in a cell line by genetic manipulation in vitro, we will use two independent approaches targeting the gene as well as a control for that approach. Moreover, we would typically examine more than one model cell subpopulation/line. Likewise, we may use several doses of a drug, or several different drugs or treatment combinations to test a hypothesis. We have estimated the total number of mice to be used over the licence lifetime taking into account our previous experience in PPL PDCC31AAF.

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

number of animals being used in this project?

The use of mice will be minimised in several ways:

By considering on-going statistical estimation of power requirements in each of the studies, using prior results in order to use the minimum number of animals while retaining sufficient numbers for statistical significance. In general, we will use a sample size capable of detecting a 40% practical difference with 80% power and 95% confidence.

By incorporating as many test groups as possible within a single controlled experiment, reducing the number of controls required compared to a series of smaller experiments.

By utilising tissues and tumours from different sites on one mouse for both treatment and control samples.

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

The use of mice will be minimised in several ways:

By optimising our breeding programme to maximise use of mice in experimental protocols minimising the number of mice we do not require. Breeding is performed in a different PPL, by a dedicated group in our Establishment.
By doing as much preliminary work as possible in culture models in vitro and in silico analysis prior to engaging in in vivo studies.

By minimising variability in results through utilising inbred strains and by housing them under identical conditions to limit variability.

By performing pilot studies using few mice when no information is available in the literature so that the number of mice utilised in experiments is reduced to minimal levels.

In all new experimental models and protocols, we will establish the base line by procuring help and advice from BRU staff and researchers at the Establishment but also from our experienced collaborators outside across the UK and internationally. Furthermore, we will design small pilot experiments, carried out referring to https://www.nc3rs.org.uk/conducting-pilot-study, that will allow us to select the ideal cell line so fewer animals are used, to calculate the minimum cohort size given the rate of expected events and also to determine gravity of these, allowing more accuracy for statistical powering calculation of group sizes in potential repeats. Lastly, pilot experiments, potentially for all aspects of the Protocols, will enable us to better plan the length and size of the experiment and to help monitor for side effects at the critical time points.

Refinement

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

Which animal models and methods will you use during this project? Explain why

these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The use of inbred and fully back-crossed mice not only reduces intragroup experimental variability but also eliminates incompatibility when cell transfers are carried out between various knockout, transgenic and wild-type strains.

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

The cancer mouse models that we will use very closely recapitulate the human disease and thus allow to understand the molecular and cellular events and steps involved in the activation of tumour immunity and tumour-related inflammation during tumour development and progression. Some mice in which we are investigating the effects of treatments upon the spread and progression of tumours (up to 3% based on recent experience) will be unexpectedly found dead over night when monitoring indicates that they are normal. This is due to a sudden impact of the spread of cancer cells from the original location to other body organs. The health of all mice will be observed daily. We believe we can justify this because we have seen unprecedented outcomes with our approaches in multiple malignancies, including some once thought to be treatment refractory.

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

Where possible, procedures will be undertaken under anaesthesia with the administration of analgaesia to minimise the experience of pain.

We constantly work to improve husbandry and procedures to minimise actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare. Mice will be maintained in individually ventilated cages under barrier environment, to avoid infections.

When considering which route of administration of substances to employ, we will strive to use the least invasive route whilst maintaining direct control of dose. The choice of route to administer a drug or cells will be such as to achieve "best practice", i.e. to minimise or avoid adverse effects, reduce the number of animals used, and maximise the quality and applicability of substances and cells to achieve the scientific objectives.

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

Surgical procedures will be carried according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

Unless otherwise specified, this project will follow the "Guidelines for the welfare and use of animals in cancer research" and the administration of substances and withdrawal of blood will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm (Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555-77 (2010)).

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

By reading 3Rs literature and participating in 3Rs workshops locally and nationally.

Through discussing refinements with our NACWO, NVS and ASRU. I am a regular attendee and contributor to our Retrospective Review and Licensees meetings and the 3Rs Poster session, all of which take place annually at our Establishment.

32. Pharmacology and physiology of CNS neurotransmission

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Psychiatric disorder, Neurotransmitters, Drug therapy, Risk factors

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Neurotransmitters are the brain's main signalling molecules. The overall aim of this programme is to utilise animal models to gain new knowledge on the pharmacology and physiology of brain neurotransmitters, and to use this knowledge to help make advances in the treatment, prevention and detection of individuals at risk of poor mental 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?

Mental illnesses such as depression, anxiety and schizophrenia are common, seriously disabling, and are linked to self-harm and even loss of life through suicide in a significant number of sufferers. Despite this, even the most modern drug treatments do not achieve useful therapeutic effects in all patients and often have a delayed onset of action of many

weeks. Moreover, these treatments can cause unwanted side effects including when patients try to stop their medication, and some of the drugs have been linked to later life problems in the offspring of mothers who are prescribed drugs to treat mental health problems during pregnancy.

One of the main reasons for all this is that we still don't fully understand how the drugs work in the brain or what makes individuals vulnerable to the mental illnesses that they are aimed to treat. In particular, there is a need to increase knowledge of the neurotransmitters that underpin psychiatric symptoms and their subsequent relief by treatment. With increased knowledge of brain neurotransmitters drug treatments could be developed rationally rather than by empirical means as at present, and safer treatments could be prescribed with a prior knowledge of whether they will work in a particular patient or not.

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

Our data will contribute to new knowledge on the pharmacology and physiology of key neurotransmitter systems and complex brain microcircuits, and how changes in these neurotransmitter and microcircuits bring about changes in emotion and cognition.

We will generate data that will speed up the development and design of future drug therapies that will be more effective than current drug treatments of psychiatric disorder and with fewer problematic effects such as potential harm to the developing brain.

Our data will reveal new information on the actions on brain microcircuits of environmental and genetic factors that put individuals at risk of developing psychiatric disorders such as depression and anxiety.

Through collaboration with computer scientists our data will generate computational models of the functional connectivity of key microcircuits relevant to psychiatric disorders. These models will help make a much more complete explanation of how microcircuits are changed by genetic and environmental risk factors and acted on by drug therapies.

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

New understandings of neurotransmitters and brain microcircuits will be of interest to a large community of researchers in many disciplines including neuroscience, pharmacology, psychology, psychiatry and computational neurobiology.

New data on drug development and design will benefit industry that is making considerable investments in new drug therapies. Also, it will be helpful to clinicians who are treating patients and developing prevention strategies. It will also be of value to patients and their carers themselves, who are trying to understand and cope with psychiatric disorders.

New information on the mechanism of psychiatric disorder risk factors will be helpful to clinicians who are treating patients and detect those at risk. It will also be of value to patients and their carers themselves, who are trying to understand and cope with psychiatric disorders.

Developments in computational biology will have benefit the 3Rs in terms of reducing and replacing animal use.

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

We will continue to maximise output from our programme of work in a number of ways:

Contribute to the UK knowledge economy through timely publication of high-quality research accessible to academics and clinicians.

Maintain and develop links with local research groups and clinicians to explore how our findings can inform potential drug discovery and clinical practice.

Maintain and develop existing links with the pharmaceutical industry.

Contribute to the economic competitiveness of the UK through enhancement of researcher career development and the training of young scientists.

Develop an animal research framework that reduces the number of animals required through maximising data yield.

Promote greater public understanding of science through continuation and expansion of our current public engagement activities.

Species and numbers of animals expected to be used

- Mice: 9,000
- Rats: 100

Predicted harms

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

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

We need to use animals (mainly mice but also rats) in our research programme for several reasons:

The rodent brain is a simple model of the human brain but significant advances in our understanding and treatment of psychiatric disorders are under-pinned by studies on small animals.

The rodent brain is the most investigated of all vertebrate brains and provides an incomparable database to aid our investigations.

Our studies on neurotransmitters are focused on complex neural pathways that cannot currently be modelled without animals.

Emotional and cognitive responses are complex behaviours with human correlates and these cannot be studied in non-sentient animals.
The influence of genetic and environmental (eg. developmental) factors on emotional responses and cognition cannot be modelled in vitro or by computers.

By using animals we can administer pharmacological agents at doses and over intervals that have direct clinical relevance. The relevance of drug doses in in vitro preparations to doses experienced by patients is often uncertain.

We will utilise adult animals but also animals at earlier life stages because we wish to study brain development, a complex process which cannot be modelled in other ways.

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

We will manipulate brain neurotransmitters in a number of ways including the use of animals with genetic modifications and novel optical and chemical approaches that allow manipulation of neurons with a high degree of control.

We will make molecular and cellular measurements in post-mortem tissue as well as measurements of neuron activity in vivo.

Behavioural analysis will also be carried out and include measurement of natural emotional and cognitive responses that are highly relevant to symptoms of psychiatric disorder.

We will use a social defeat paradigm as a model of environmental stress because of its good construct, face and predictive validity. Social defeat is a natural stressor in animals, and a source of depression, anxiety, low self-esteem, and social withdrawal in humans.

Some experiments involve the surgical preparation of animals under general anaesthesia, for example to deliver genes to the brain or for implantation of microelectrodes or other miniature recording devices. On occasions animals will be treated for several weeks with drugs, the majority of which will have proven tolerability and safety in animals (and humans).

At the end of the experiments animals will be killed by humane methods.

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

Adverse effects associated with these surgical approaches are rare but range from wound breakdown and infection to a build-up of fluid on the brain, which may result in neurological signs. Appropriate surgical methods (eg. aseptic conditions) and post-operative care (eg. pain-relieving drugs) will be used to minimise adverse effects such as neurological signs.

Surgically prepared animals will be monitored to detect signs of adverse effects before they fully emerge and thereby allow us to adjust our experiments to reduce their frequency and severity.

Some drugs may cause short-lasting behavioural stimulation or sedative effects and will be administered using doses that produce effects of the smallest magnitude appropriate to the experiment. Animals administered drugs will be frequently monitored and weighed, and if such adverse effects are observed they will be resolved by adjusting the dosing regimen. In certain behavioural tasks animals will be food-restricted to ensure that they are motivated to perform the task. These animals are generally healthier than animals with unrestricted access but we will carefully monitor for excessive weight loss and use endpoints that trigger supplementary feeding to return weight to acceptable levels.

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

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

Mice; 50% sub-threshold, 25% mild, 25% moderate.

Rats; 50% subthreshold, 25% mild, 25% moderate.

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

- Killed

Replacement

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

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

We need to use animals (mainly mice but also rats) in our research programme for several reasons:

Our studies on neurotransmitters are focused on complex neural pathways that cannot currently be modelled without animals.

Emotional and cognitive responses are complex behaviours with human correlates and these cannot be studied in non-sentient animals.

The influence of genetic and environmental (eg. developmental) factors on emotional responses and cognition cannot be modelled in vitro or by computers.

By using animals we can administer pharmacological agents at doses and over intervals that have direct clinical relevance. The relevance of drug doses in in vitro preparations to doses experienced by patients is often uncertain.

In some experiments we will utilise animals at earlier life stages because we wish to study the brain as it develops, a complex process which cannot be modelled in other ways.

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

Our animal work is complemented with studies on neurotransmitter signalling in cultured cells and we collaborate with computational biologists to analyse, understand and model large complex datasets.

Why were they not suitable?

It is not possible to model complex neural circuits, emotional and cognitive responses, or brain development in cultured cells.

Computational approaches have not yet proven to accurately model the functions of integrated living brain circuits and their complex functions. These approaches are limited by factors such as dimensionality, the availability of incomplete data, and outcomes and predictions that require testing and verification in an experimental 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 estimated the number of animals on the basis of previous experience, our technological approaches, and experimental design which take into account the following to minimise the number of animals used:

Use of good surgical methodology and practice to minimise loss of animals by maximising chance of recovery (eg. asepsis, use of short acting anaesthetics, minimal surgery time and postoperative care and analgesia).

Where possible use of in vitro experiments to generate models and hypotheses to guide in vivo experiments.

Rather than repeating controls, use of "rolling controls" for experiments spread over time.

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

We will continue to conduct our experiments so that they comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines). This includes but is not limited to:

Use methods of statistical analysis and randomised, blinded experimental designs combined with power calculations to establish the minimum number of animals required to achieve a meaningful result.

Where necessary use of alternative methods of statistical analysis to minimise the need for repeating experiments and/or maximise the conclusions drawn.

Use appropriate controls to prevent false positive/negative results (eg. use of drug vehicles, an inactive drug of similar chemical structure, or pharmacological blockade to confirm specificity of action).

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

Use of well-managed and conservative genetic mouse breeding strategies and use of both male and female mice, as well as heterozygote mice where appropriate.

Use of electrophysiological recording methods that detect many tens of neurons in a single animal, thereby reducing the number of animals required and at the same time generating large biological datasets to capture neural circuit complexity.

Sharing of datasets with collaborating computer scientists to develop computational models of key neural networks to aid further hypothesis development and prediction testing.

Use of pilot experiments before embarking on full-scale studies, and literature searches will be carried out to avoid replication of experiments already published.

Refinement

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

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

Although the rodent brain is a simple model of the human brain significant advances in our understanding and treatment of psychiatric disorders are under-pinned by studies on small animals, as exemplified by recent evidence of the dawning of a new generation of rapid-onset antidepressant drugs. The rodent brain is the most investigated of all vertebrate brains and provides an incomparable database to aid our investigations.

Our studies on the effect of drugs and psychiatric risk factors (environmental stress, genes) on neurotransmitter function are focused on complex neural pathways that cannot currently be modelled without animals. Moreover, by using animals we can administer pharmacological agents at doses and over intervals that have direct clinical relevance. The relevance of drug doses in in vitro preparations to doses experienced by patients is often uncertain.

We will also investigate neurotransmitter function and neural network formation during brain development, which is a process unique to mammals and cannot yet be modelled in vitro or by computers.

The vast majority (>95%) of drugs that we use will have known pharmacological profiles and proven safety in animals at the doses selected.

We will measure key emotional and cognitive responses with close correlates to those exhibited in healthy humans and dysfunctional in patients with psychiatric disorders. These responses also cannot be studied in non-sentient animals.

The majority of our behavioural paradigms are harmless and capitalise on natural and spontaneous behaviour to test emotional and higher cognitive functions. Anxiety paradigms are largely exploratory-based tests that generate a natural approach/avoidance conflict response. When used, rewarding stimuli are natural motivators and readily accepted.

Some behavioural paradigms will probe aversion learning and stress vulnerability and involve the delivery of an acute aversive or stressful stimulus, which is calibrated to be brief and cause no lasting harm. A large scientific literature attests to the value of using aversive/stressful stimuli to investigate the neurobiology of stress mechanisms in a controllable, reliable and refined way.

The neurobiology of stress vulnerability cannot be studied without exposure of animals to stressful stimuli. We will use an ethologically-based model that uses a natural stressor (social defeat) that is more refined than other stress models based on non-natural stressors such as inescapable physical restraint.

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

Animals less sentient than those selected:

do not have neurotransmitter systems and complex neural circuits that are representative of the mammalian brain.

cannot be administered pharmacological agents at doses and over intervals that have relevance to those experienced by patients.

are unable to perform complex emotional and cognitive tasks that model similar tasks in humans and are dysfunctional in patients with psychiatric disorder.

do not demonstrate the process of brain development that is characteristic of mammals.

have nervous systems that are much less investigated than the mammalian brain, and do not have comparable databases to aid our investigations.

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

Use of health monitoring of adverse effects with humane endpoints.

Use of health monitoring to avoid cumulative effects of experimental steps and circumvent where necessary.

Use of peri-operative analgesia which will be administered and continued after surgery for as long as required to alleviate pain.

Optimise experimental parameters to produce reliable effects with the minimum duration and frequency of treatment (eg drugs, aversive/stressful stimuli) exposure.

Use of literature and other databases to aid selection of drug treatments that produce optimal effects and minimal adverse effects. Treatments with the latter potential will be detrimental to our studies and avoided.

Drugs to be administered in volumes and routes that are the least harmful and most appropriate to the species.

Drugs with uncertain safety and tolerability will initially be tested acutely at low doses in pilot experiments.

Use of ARRIVE guidelines (https://arriveguidelines.org) to ensure the use of the most refined methodology.

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

www.nc3rs.org.uk, plus regular retrospective reviews by local ethical review committees and updates from our Named Information Officer.

guidelines for administration of substances (volumes/routes), e.g. Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn & Maggie Lloyd (2013, 4th Edition), Blackwell publishing.

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

We are on the mailing list of www.nc3rs.org.uk. We have an NC3R's regional manager available to us and we regularly attend internal 3R's meetings.

33. Helminth regulation of 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

soil-transmitted helminth, cancer, prevention, treatment, immune system

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To understand the mechanisms for how helminth (worm) infections influence the body's immune system.

This will help us to find new ways to prevent or treat important diseases they influence, such as 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?
Approximately 1.45 billion people (<20% of the world's population) are infected with one or more types of soil-transmitted helminth (STH). Infection mostly occurs within low-and-

middle income regions, including sub-saharan Africa, South-East Asia and South America. Heavy infection with STHs is associated with the impaired physical and cognitive development of school children, whereas heavy infections in pregnant mothers can result in reduced infant survival and birthweight. Overall, 5.2 million years of life are lost from a population's lifespan due to ill health because of a STH infection. Currently, there are no vaccines for disease and treatment relies on the use of mass drug administration. These drugs do not prevent people from becoming re-infected with STH and often, drug resistance develops, so people cannot be effectively treated.

From human studies and by modelling human STH infections in mice, we understand that they can establish themselves in the intestine for long periods of time. While in the intestine, they have a profound impact on the gut microbiome, our immune system, as well as our ability to harvest energy and nutrients from food. We also understand that one of the ways helminths can have these effects is through the secretion a set of molecules, termed “excretory/secretory (ES) products”. The ability of STH infection and the ES products to manipulate the immune response is linked to their ability to survive in the host and produce fertile eggs, without killing the host. This manipulation of the immune response has been shown to affect the development of other diseases, such as asthma and diabetes.

One in two people in the UK will develop cancer in their lifetime. Although the incidence of cancer is currently much lower in low-and-middle income countries (LMIC), than in high-income countries (HIC), it is anticipated to increase by 62% in LMIC, such as South Africa, when compared to an increase of 30% in HIC, such as the United Kingdom, by 2040. High levels of infectious diseases within LMIC are a risk factor for cancer development and were estimated to be the cause of 32.7% of new cancer cases in sub-Saharan Africa in 2008.

Although, STH infections have been found to be a risk factor for increased colorectal cancer in mouse models of disease and higher rates of human papillomavirus (HPV)-related cervical cancer in helminth endemic areas, our laboratory studies have linked exposure to ES products with decreased cancer. It is therefore currently not clear how STH infections and their ES products influence the development and progression of cancer, which is why we now wish to undertake this project. We will make use of established models to study the interaction between STH infection and the immune system in mice, to better understand this interaction and improve treatment strategies for the prevention and treatment of the important diseases they influence, such as cancer.

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

This project aims to generate new information on how soil-transmitted helminth (STH) infections influence our immune response and alter our risk of developing important diseases, such as cancer. This will help us identify new ways to try and treat STH infections, as well as new ways to prevent or treat cancer. This information will be shared at local and international conferences, within open access publications and through public engagement activities.

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

Within the timescale of the project, the scientific community is most likely to benefit from these outputs, through our increased knowledge of how soil-transmitted helminths influence the development of cancer. In the longer term, our discoveries aim to identify new pathways that can be targeted in order to prevent or treat disease. Through identifying

these mechanisms, the longer-term impact of developing potential therapeutics for the treatment and prevention of cancer is high.

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

I am involved in a number of academic collaborations within the field (across several institutions within and outside the UK, including in relevant helminth endemic countries, such as South Africa). In addition, I have ongoing collaborations with clinicians in the field of colorectal cancer. 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 attendance and presentation. This will continue across the life of the current project - supporting the wide dissemination of the outputs from this work. I am committed to the communication of science to the public (including invitation to speak at Soapbox science events, as well as Pint of Science events). I am further committed to "open science", through the communication of validated unsuccessful approaches within appropriate channels.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 200

Predicted harms

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

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

The soil-transmitted helminths we study can only complete their life cycle in living animals, in our case mice and rats. In addition, we and others have experience of using well-established mouse models of helminth infection, that accurately reflect infection in humans. Similarly, we and others have experience of using well-characterised mouse models of cancer, that reproduce human disease and allow us to study the influence of helminths on cancer development. Due to the complexity of the interactions between live intestinal helminth infections and tumour development in the body, there is currently no way to recreate these conditions in the laboratory. Our studies will use adult rodents, which can independently feed and have a fully developed immune system, as these most appropriately model the life stage of people infected with helminths, or who develop cancer.

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

In order to generate the helminths we need to study their interaction with the immune system, we will infect mice and rats with helminths so that we can grow the helminths and collect excretory/secretory (ES) products from them. These animals will be injected, or given helminths by mouth, just once.

In order to study how these helminths influence cancer development, a separate cohort of mice will be injected with tumour cells or agents that cause cancer, using the following

established models with a known time course. Approximately half of these animals will also be exposed to helminths by infecting them, or by injecting helminth secretions. Experiments will last 2 weeks up to 4 months.

Genetically altered mice will be given an agent that causes genetic changes that lead to cancer, similar to those seen in humans, either in their food or water, into abdominal cavity or under the skin of the colon, while under anaesthesia for a short time. Tumour growth will be monitored using in vivo imaging under anaesthesia for a short time, or at the end of the experiment, which will last a maximum of 4 months.

Animals will be injected with an agent that causes genetic changes that lead to cancer, similar to those seen in humans, into the abdominal cavity. They will then be given an agent that causes inflammation in the intestine for a period of one week, every fortnight on a total of 3 occasions, in their water. Tumour growth in the colon will be monitored using in vivo imaging under anaesthesia for a short time, or at the end of the experiment. Following injection of the agent that causes genetic changes that lead to cancer, these experiments will last a maximum of 10 weeks.

A cohort of female animals will be given a human papillomavirus virus (HPV)-like particle while under anaesthesia for a short time. Approximately 10% of mice will be vaccinated with the HPV vaccine Gardasil, before delivering the particle. The vaccine protects humans from infection with four different types of HPV, which cause almost all cases of cervical cancer. Infection with HPV will be confirmed by imaging the mice under anaesthesia for a short time. After the imaging sessions are complete, within 72 hours following human papillomavirus infection, the animal will be humanely euthanised.

In some mice, tumour cells will be injected a) under the skin while the mice are awake, b) at one site into the wall of the lower intestine (e.g. colon), while anaesthetised for a short time, or c) into the blood. Tumour growth will be monitored using a hand-held calliper measuring device in mice that are awake, or by in vivo imaging under anaesthesia for a short time, up to maximum of 3 weeks.

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

Most animals show no untoward effects of the helminth infection itself in the doses and time scales we use. Rats and mice infected with certain helminths will develop transient weight loss, which is associated with the natural lifecycle of the helminth (as it passes through the lung). We will monitor these animals for weight loss following infection, for a maximum of 9 days. If an animal loses >15% of their starting body weight, they will be humanely killed.

To test the effects of helminth infection on cancer development, some animals will develop stressful signs associated with cancer, including tumour growth, weight loss, intestinal discomfort or diarrhoea, depending on the model used. We will monitor tumour growth for a maximum of 4 months, or until the tumour reaches a predefined size, which we have set to avoid it causing any adverse effects to the animal. In addition, we will use our score sheet to monitor the clinical signs of cancer, for a maximum of 4 months. If an animal reaches a predefined clinical score, or they lose >15% of their starting body weight, they will be humanely killed.

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

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

Mild - 100% of rats, 75% of mice. Moderate - 25% of mice

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

- Killed

Replacement

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

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

The soil-transmitted helminths we study can only complete their life cycle in living animals, in our case mice and rats. Infection causes prevalent diseases in humans and livestock and is a risk factor for cancer. The complex interactions between live intestinal helminth infections, the host immune system and tumour development depend on a complex integration of signals, which can only be found in the live mammalian animal.

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

To date, we have established how helminth secretions influence cancer cell behaviour, using cancer cell lines. We have considered this alternative for this project, as well as laboratory cultivation of helminths without the need to infect live animals, as well as the use of tissue explants.

Why were they not suitable?

Currently, it is not possible to cultivate soil-transmitted helminths to complete their life cycle entirely in the laboratory, although this may become possible in the future and we will continue to monitor progress in this area. Interactions between live intestinal helminth infections and tumour development in the body are complex and depend on the integration of signals from the microbiota, tissues and the immune system. Cell lines and tissue explants are limited as they currently do not recreate this complex web of interactions.

Reduction

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

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

In order to estimate the numbers of animals we will use in order to achieve the objectives of this project, I have referred to the annual usage from my own research over the past 18 years, and that of relevant colleagues, having considered where the numbers of animals used could be reduced. In addition, data generated in my laboratory with cancer cell lines

and my experience with the murine models of cancer herein has informed the minimum numbers of animals required to achieve statistically significant findings.

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

I have attended courses on experimental design and statistical approaches and have sought further statistical advice from colleagues whenever necessary.

Data generated in my laboratory with cancer cell lines has informed the minimum numbers of animals required to achieve statistically significant findings. In addition, the experience I have had with these murine models of cancer as a research fellow over the last 7 years, has allowed a prediction of the experimental variation we should expect.

Our previous experience with these models has also informed our ability to reduce the number of animals used. In all experiments, in-bred mice from the same litters will be used in order to reduce variation. Should we need to use differing litters, genetically altered mice or transgenic animals, we will mix the bedding of all mice and allow their microbiome to stabilise for 2 weeks, in order to reduce variation that can occur due to differences in the microbiota.

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

Our studies will make use of known models of disease and helminth infection and will make use of methods like imaging in order increase the amount of data obtained from single animals.

My group will take the approach of performing pilot experiments, in order to determine the optimal number of mice to achieve statistical power. Experiments will then be 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.
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models we use for helminth infection cause minimal suffering or distress to the rodents as they are naturally well-adapted to their host. The mouse models for cancer do inescapably cause a degree of suffering as they are designed to replicate human disease. Our approach is to select models which are very well established, well characterised and accurately model the course of human disease. In addition, we will regularly monitor and score animals based on the clinical signs indicative of disease, in order to select a window of time for analysis which minimises any suffering the animals may experience.

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

The lifecycle of the soil-transmitted helminths we use has been developed in adult laboratory rodents, and no alternative host species is known. The animal models for cancer have also been developed in adult laboratory mice. As adults, these animals can feed independently and their immune system is fully developed, representing the stage at which humans become infected with helminths and develop cancer. Helminth infection can take up to 4 weeks to establish and cancer can take up to 4 months to develop, therefore it would not be possible to use animals that have been terminally anaesthetised.

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

We will minimise the welfare costs for the animals by following the guidance of the ARRIVE (Animal Research Reporting of In Vivo Experiments) guidelines, available through the NC3Rs website. We will also refer to the advice of our Animal Welfare and Ethical Review Body (AWERB), which produces specific guidelines that further refine and ensure best practice.

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

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.

We also regularly consult the nc3rs refinement resource website (https://www.nc3rs.org.uk/3rs- resources), including their webinars, training material and other e-learning resources, in order to ensure experiments are conducted in the most refined way.

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

We will continue to discuss published advances in the 3Rs at regular laboratory meetings and through discussion with our Named Animal Care and Welfare Officers (NACWO) and Named Veterinary Surgeon (NVS). In addition, we will remain informed about advances in the 3Rs, including NC3R symposia, through our local named persons and our NC3Rs Regional Programme Manager.

34. Placental epigenetic programming

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Placenta, Epigenetics, Development, Gene regulation, Embryos

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to gain an understanding of the importance of early events in genome regulation in the development of a healthy placenta during 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?

Pregnancy complications such as preeclampsia, miscarriage or preterm birth affect as many as one in four pregnancies, yet there remains a critical gap in our knowledge of the underlying causes. The placenta not only supports the growth of the baby, but helps the mother’s body adapt to pregnancy. A poorly functioning placenta can therefore not only impact the survival and health of the baby, but in many cases the mother as well. At present, we have a poor understanding of early events that lead to healthy placental

development because these developmental milestones occur during the first weeks of human pregnancy.

Evidence supports that pregnancy complications are linked to changes in genome regulation in the placenta. In particular, epigenetics marks (chemical changes to DNA or its bound proteins) are key to setting up the active and silent regions of the genome during development. The patterns of epigenetic marks are unique to each cell type in the human body. Importantly, the establishment of these patterns are critical for a tissue to form correctly and function.

We want to understand the patterning of epigenetic marks in early placenta development. We hope to reveal how these marks direct the formation of a functional, healthy placenta, using the mouse model. Critically, mouse and human placentas contain similar cell types, epigenetic patterns, and developmental milestones, making the mouse a suitable model for this research.

We hope that our research over the next five years will lead to an understanding of how epigenetic patterning of placental cells in the early embryo support healthy placental development throughout pregnancy. This work will lay the foundation for our understanding of changes that may underlie complications of pregnancy.

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

The main outputs from this project will be a better understanding of the importance for epigenetic marks in directing placental development. We will share our findings through presentations at national and international conferences, publication in peer-reviewed scientific journals, and exhibitions at public outreach events, such as science festivals. Alongside publication, all datasets generated from our projects will be shared with the scientific community in public data repositories.

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

In the short term, these studies will provide important discoveries of the processes involved in early placental development. Our findings will help both clinical and basic science research in reproductive biology and epigenetics to generate new research questions. This work will form the basis for future research funding applications. These studies will also provide sequencing datasets from the early stages of mouse embryo development, when cell types are first being established. These available datasets can be further explored by other research groups to address fundamental research questions.

Importantly, these studies will help work towards an understanding how molecular changes early in development may lead to placental dysfunction and complications of pregnancy in humans. At present, there is a lack of clinical tools to provide early detection to help improve outcomes for complicated pregnancies. Hence, obtaining a better understanding of the underlying contributors to pregnancy complications will be a crucial step towards better supporting these pregnancies. In the long term, this work will help the public through improved care options for mothers and babies during pregnancy.

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

The outputs of this work will be maximised, in part, through collaborations. With a strong collaborative team, we will ensure the successful use of novel techniques and strong study designs. We will publish in high-impact, open-access journals, allowing us to widely

distribute the knowledge generated by our work. We will publicise major findings in the press to further improve visibility of the research. When possible, we plan to publish negative results and detailed methodologies in open-access journals.

Species and numbers of animals expected to be used

- Mice: 4900

Predicted harms

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

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

We are using the C57BL/6 inbred strain of mice because this is the most common background for genetically modified lines of mice. C57BL/6 is the best known mouse genome for mapping of genomic sequencing data. The use of C57BL/6 will allow our data to be comparable to other studies in the field.

We have chosen to evaluate several stages of embryo development after the embryo implants into the mother's uterus, as this is when placenta formation and function begins. We will assess epigenetic marks, gene expression and placental structure at various critical stages for placental biology. First, we will assess embryonic days 4.5 to 7.5 because at this time the embryo contains stem cells that will later derive all of the placental cell types and coincides with the patterning of epigenetic marks. We will then assess embryonic day 9.5, which is a critical time point in mouse placental development, when the cells that generate the umbilical cord link the baby to mother. Finally, we will evaluate embryonic day 12.5 because at this stage the placenta is fully formed, containing all of the necessary cell types for its function.

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

For animals used for this project, no procedures other than observational handling and matings are planned. Mated female animals will be humanely killed at predetermined life stages for embryo and placenta collections. Tissues will be used for generating genomic sequencing datasets to investigate genome regulation and for analysing cellular and structural changes in the placenta.

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

We do not expect adverse effects on the animals during this project because we plan to assess mice with a loss of gene function only at embryonic stages. There are no plans to evaluate these animals past birth.

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

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

Mice Mild 100%

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

- Killed

Replacement

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

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

Studying the earliest stages of placental development in humans is logistically and ethically challenging because these events occur during the first few weeks of pregnancy. The experimental tools to study embryo implantation in the lab have not yet been developed. Hence, the mouse model provides a mammalian system in which we can study the molecular events during the formation of the placenta.

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

Human placental (trophoblast) cells can now be grown in lab, so we considered whether this would be a good alternative for this project.

Why were they not suitable?

Culture methods for human placental trophoblast cells are very new and still under development. As a result, these methods are not yet able to accurately model the early developmental windows of the placenta. Importantly, culture methods also cannot yet recreate the unique environment of the placenta. The placenta forms at the interface between mother and baby during pregnancy and contains cells from both mom and baby. Hence, the study of mouse development is essential in furthering our understanding of key steps in early placental development.

Importantly, prominent molecular features, such as the pattern of epigenetic marks on the placental genome are conserved between mouse and human. Mouse and human placentas have similar cell types, developmental milestones and cellular structure to separate the blood of mother and baby. As a result, our studies in mice will provide a critical foundation for future research into important regulatory events in human placental development.

Reduction

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

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

We have prioritised investigating two epigenetic pathways in placental development. We will be studying knock-out (carrying two copies of a genetically modified gene) mouse models for the key enzymes in each of these pathways, which includes a total of 7 mouse lines. We estimate that each line will require 700 animals over 5 years to allow for enough matings to maintain the lines and perform experiments.

Maintenance of these mouse lines will be done by mating animals carrying a single copy of the genetically modified gene (heterozygous) to unmodified animals. This strategy is optimal for ensuring animal wellbeing and health. For each experimental mating, heterozygous animals will be mated together to obtain embryos. Embryo tissues will be used to generate molecular and sequencing datasets and to perform characterisation of the placental and embryonic features. Important considerations in this estimation of animal numbers are the frequency at which the correct genotypes can be obtained, the number of developmental time points being assessed in each line, and the number of experiments to be performed.

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

I have used the online experimental design tool provided by NC3Rs and the PREPARE guidelines to optimise of my study design, bias avoidance and sample sizes. I plan to collect embryos from heterozygous female animals bred to heterozygous male animals. This heterozygous breeding strategy allows the collection of control and knockout embryos from a single pregnant female. Furthermore, using this approach, the genetic status of embryos is unknown to the experimenter during collection.

The genetic status of each embryo is determined after samples are frozen or fixed, minimising any collection bias. Using Power Calculations, I have determined the minimum sample size needed for comparison of genomic sequencing datasets to be 3-5 per group. This will allow us to confidently identify changes in gene expression and/or epigenetic changes that are likely to impact cellular or tissue biology. Samples will be randomised for all molecular experiments to avoid technical bias.
Evaluating cellular and structural changes in the embryo and placenta will be done using 5 samples per group to identify large, easily observable changes because we expect features to be dramatically altered in knockout embryos. The genetic status of each tissue will be unknown for experimenters assessing placental structure and cell composition.

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

My collaborators and I have developed cutting-edge adaptations of all sequencing methods. Rather than millions of cells required by the original protocols, we have methods that now only require hundreds to thousands of cells to obtain high-quality data. Without these advances, this project would not be feasible in mouse models.

In my postdoctoral work, I generated similar types of sequencing data from eggs and embryos collected from genetically modified mouse models. I also have performed pilot experiments in mouse embryos to demonstrate the quality and reproducibility of data that can be obtained from placental trophoblast cells. These studies have helped me accurately estimate the number of animals required to achieve our research goals.

The research project is designed with staggered aims, first focusing on one specific epigenetic pathway, followed by the other. This approach minimises the use of animals by avoiding unnecessarily prolonged breeding.

Refinement

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

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

Our plan is to use genetically modified mouse lines for breeding and maintenance. We don't plan any procedures or protocols that will cause pain, suffering or distress. Animals carrying the genetically modified targets selected for this study do not show any adverse phenotypes. Knock-out animals will be evaluated before birth.

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

To study placenta formation in pregnancy, it is necessary to study placental mammals. Hence, the mouse model is the least sentient model that we could use for these studies.

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

Stud males are those that are temporarily mated with females to obtain embryos at a specific stage. After pairing with a female, stud males cannot be returned to cages with other males, as this often result in fighting and aggression-related injuries. Hence, these males will be temporarily housed individually between matings. To ensure the wellbeing of these animals, we will singly house stud males for the minimum necessary time and utilise several refinement approaches, including the use of enriched environments and acclimatisation/rest periods between matings. To ensure the wellbeing of pregnant females after mating with stud males, females will be housed together until the relevant predetermined life stage.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
For all animal work, I will follow the Laboratory Animal Science Association (LASA) guiding principles to ensure refined and considered protocols and approaches. I will adhere to guidance provided by NC3Rs strategies and PREPARE guidelines. I will frequently liaise with our Named Animal Care and Welfare Officer and Biological Support Unit facilities to review refinement of our approaches. We also plan to attend events and meetings, such as those provided by my organisation and NC3Rs, to stay updated on best practices.

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

We will stay up-to-date on advances in the 3Rs from the National Centre for the 3Rs (NC3Rs) guidelines and updates, as they evolve through the duration of the project. We will also use the Norecopa resources and University of Cambridge 3Rs search tool to find recent articles and relevant 3Rs news. We will stay attuned to the latest practical guidance from Laboratory Animal Science Association (LASA). In particular, we will closely follow any updates on the refinement and usage of genetically altered mice.

35. Epigenetic regulation in 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

Cancer, Epigenetics, Chromatin, Immunotherapy, Development

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Decipher epigenetic pathways in mouse models of oncogenesis to understand fundamental mechanisms and develop effective therapeutic strategies

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

Why is it important to undertake this work?

Normal development in mammals is driven largely by reversible and heritable modifications to DNA, RNA, and protein that do not alter the sequence information. These marks and their precise regulation of gene expression make up the field of epigenetics. These mechanisms work through widespread labelling with diverse chemical modifications and work in complex combinatorial ways through tightly regulated activating and repressive signals. Such regulation within our cells is dynamic and finely tuned through the

enzymatic action of epigenetic modifiers that add or remove these marks, known as “writers” and “erasers”, respectively. Many of these modifiers target protein tails extending off of core proteins around which DNA is wrapped. This DNA/protein structure referred to as chromatin resembles beads on a string under an electron microscope and it organizes our genome in the nucleus and determines how regulatory proteins are able to engage and read our genes. These protein tails can be labelled with many distinct marks and it is the interplay between these that regulates gene expression. Such broad capacity for fine-tuned control extends to DNA modifiers and over 100 different types of RNA modifications. When these exquisite regulatory mechanisms fail or become dysregulated, a wide array of developmental diseases, autoimmunity, ageing and cancer can develop. The work under this application will allow us to understand these aberrant epigenetic mechanisms and discover ways to reverse or prevent their occurrence in order to improve human health through the development of effective therapies.

In part, our aim in understanding fundamental epigenetic mechanisms is focused on two fronts. These are age-associated loss of immune function known as immunosenescence and cancer.

Age-associated epigenetic dysfunction leads to many disease states. This concept is perhaps most widely known by the public with DNA methylation clocks. These clocks accurately reflect the accumulation of aberrant DNA modifications with age in many animals. Such dysregulation is exemplified by excessive modifications to tumor suppressor genes, which serves to turn off the expression of these critical genes potentially leading to cancer. Age related epigenetic dysfunction also leads to immunosenescence, a state of immune cell dysfunction where T cell levels decline and immune cells lose effector or cytotoxic functions. This process drives many age-related disease states such as autoimmune disease, cancer and increased risk of infection. Our group hopes to better understand the epigenetic mechanisms driving this aberrant regulation through functional studies in immune cells from aged mice.
In addition to promoting the onset of cancer, epigenetic dysregulation allows cancer cells to rapidly evolve and evade immune detection through mechanisms that diminish tumor antigenicity and drive immune cell dysfunction. This is exemplified by “cold” tumors, which are characterized as having very few or completely absent infiltrating T cells. Several mechanisms have been shown to cause this lack of T cell infiltration including limited tumor antigen and the presence of suppressive immune cells within the tumor.

In addition, epigenetic mechanisms in immune cells can enforce a diminished state of functionality, such as that observed in exhausted T cells where chronic activation by tumor antigens and inhibitory signals on cancer cells essentially stop the antitumor T cell response. Through our research we endeavor to better harness the immune system to combat cancer through developing treatment strategies that rejuvenate or enhance immune activity for more effective cellular immunotherapies.
Using the immune system to fight cancer has demonstrated decades of success however only a minority of patients benefit from this treatment. With our work in mouse tumor models we are aiming to identify epigenetic mechanisms both in cancer and immune cells that lead to improved immunotherapy efficacy.

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

This project will provide significant benefits both in expanding our understanding of fundamental epigenetic mechanisms and in developing improved cancer immunotherapies that can move to clinical trials. In addition, any novel insight into these critical regulatory

mechanisms will be published and shared with the broader research community to advance our collective understanding.

Much of our work will revolve around one form of immunotherapy known as T cell transfer therapy or adoptive cell transfer (ACT), a cancer treatment harnessing a patient's own T cells to fight cancer. By targeting specific epigenetic enzymes in T cells collected from mice, we expect to address some of the current obstacles currently limiting the efficacy of this treatment. We expect that this will lead to significant improvements in tumor reduction and tumor clearance when infused into tumor bearing mice. These results will guide future research decisions, reach publication and potentially lead to clinical trials.

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

The scientific advancements that emerge from our work will ultimately benefit patients that perhaps currently do not have effective treatment options for unresponsive or "cold" tumors. The benefits of our work will extend beyond patients with disease by revealing new insights into epigenetic mechanisms that can be leveraged to develop effective preventative interventions for high risk factors and will reveal new therapeutic targets for pharmaceutical companies to develop epigenetic inhibitors.

More immediately, our work will lead to publications that will benefit the principal investigator and the research group through future funding awards. New biological insight from this project will improve our understanding of these fundamental mechanisms and will impact future work both in our research and for the broader research community. Much is currently unknown regarding the critical role of epigenetic regulation in these disease states and therefore a real need exists for this work.

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

Epigenetic therapies that improve mouse T cell functionality in our preclinical models will be tested with in vitro based methods in human T cells to measure how our results translate into human therapies and whether future work or clinical trials is warranted. Novel biological data and all methods/approaches will be published and/or shared with the research community through presentations, online databases and collaborations, regardless of the experimental outcome. Collaboration with other experts will be central to maximizing successful research outcomes and disseminating new knowledge.

Species and numbers of animals expected to be used

- Mice: 7500

Predicted harms

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

Explain why you are using these types of animals and your choice of life stages.
Our research draws extensively on mouse model systems. Many of the mechanisms that we study are conserved from humans to mice making this a vital part of our work. For instance, we can study the safety profile and efficacy of a new epigenetic inhibitor, develop a new strategy for expanding an immune cell subset or decipher a novel epigenetic

mechanism crucial for healthy development in mice to reliably understand how these same conserved processes work in humans.

Part of our work involves studying the interaction between tumor and immune cells and the function of these immune cells, particularly adaptive T cells, necessitating a vertebrate animal model due to their absence among invertebrates. With mouse tumor models we can study epigenetic mechanisms that drive these processes and successfully translate key findings to clinical studies because of existing mouse/human epigenetic and immunological conservation, which is not available with less sentient animal models such as fish. Mouse models are widely used in cancer research and have provided decades of important insights into tumor immunity.

Genetically modified mice are utilized in our work for studies of specific epigenetic factors, in vitro activation of T cells, and tumor studies. Using a mouse model with T cells deficient in a critical epigenetic enzyme, we are able to study how the absence of this factor increases T cell antitumor functionality.

Due to age related immune decline, 8-10-week-old mice with normal peak immune health (wild type) are often used for our tumor studies and age matched mice are used in control groups.

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

We will be using mouse tumor models, to study the interaction between cancer and immune cells. These models are transplantation models where cancer cells are injected into mice of the same genetic background as the donor mice. Much of our research will revolve around improving a form of cellular immunotherapy known as adoptive cell transfer (ACT) therapy. Broadly, we will be isolating CD4+ (helper) and CD8+ (cytotoxic) T cells from donor mice tumors, referred to as tumor-infiltrating lymphocytes (TIL). After epigenetic reprogramming and expansion in culture, these TIL will be infused into recipient tumor-bearing mice to study how various epigenetic perturbations impact T cell antitumor functionality.

Typically, the steps involved in our TIL-ACT study include tumor induction in donor and recipient mice, with the latter receiving the modified T cells collected from donor mice tumors. Tumor induction is performed with a single injection of cancer cells directly under the skin in both groups of mice and tumors generally develop for four to six weeks. When tumors reach 1000-1200 mm^3 in donors, the mice are euthanized and tumors are collected. T cells are subsequently isolated from these tumors, expanded and epigenetic factors are targeted for manipulation to improve functionality. Recipient mice with tumor volumes generally within 150-500 mm^3, receive a single intravenous infusion of T cells into the tail vein. The treatment efficacy is measured by changes in tumor volume quantified every 2-6 days (dependent on tumor cell growth kinetics or doubling time) until there is either complete remission (no measurable tumor) or a humane endpoint is reached, at which point, the mouse will be euthanized.

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

Mice are expected to develop tumors. These tumors are expected to grow for up to 6 weeks. As the tumour grows the skin around it may be affected with redness, soreness, peeling and scabbing, which will be allowed to persist for up to 14 days. Tumors may also

ulcerate (display a small round crater) towards the later stages of growth and animals showing ulceration will be humanely killed upon detection.

Animals will experience mild and transient pain and distress from brief restraint and an accompanying intravenous injection into the tail vein.

Animals may experience mild gaseous anaesthesia lasting no longer than 10 minutes during subcutaneous injection of cancer cells. Recovery is expected to be uneventful and swift.

Mice undergoing Adoptive Cell Transfer (ACT) may experience a mild form of autoimmune disease. The mice may exhibit depigmentation of the extremities which may last up to several months. A more severe response may be mild respiratory distress presenting as laboured breathing or lethargy which, if not resolved in 24 hours, will lead to the animal being humanely killed. Mice showing immobility or not responding to touch will be humanely killed upon detection.

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

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

90% Mild

10% Moderate.

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

- Killed

Replacement

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

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

Mouse models allow us to study complex systems that cannot be properly modelled with other research tools such as cell culture. Being able to recapitulate the complex interactions between cancer and immune cells in a controlled way allows us to study the fundamental epigenetic mechanisms behind this interplay. Much of the epigenetic dysregulation driving tumorgenesis and immune evasion/suppression is shaped by signals in the tumor microenvironment (TME) and currently there are no alternative models outside of animals that can replicate these complex signals. With mouse models, we can implant tumors and study how epigenetic factors modulate the immune response and we can develop effective immunotherapy strategies that augment this response. Mice are a powerful resource in cancer research due to their extensive physiological/molecular similarities to humans, the availability of many different cancer models that accurately recapitulate features of human tumorgenesis, and the decades of acquired biologically relevant knowledge and genetic resources. To understand the fundamental mechanisms

driving these processes, we must first model them effectively, and mouse models provide that ability and therefore help bridge the gap between cell culture and cancer in humans.

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

Developing 3D tumor models in cell culture as a replacement for some mouse tumor experiments was considered. In addition, our T cell expansion in culture protocol has been optimized for maximum expansion numbers requiring less donor mice for our experiments. Our culture methods and experimental assays are continually evaluated for improved efficiency and effectiveness, which leads to less mice needed for a study.

Epigenetic targets are screened in culture for improved T cell functionality before being tested in mice. Through functionality assays that measure important aspects of effective ACT such as T cell proliferation and cytotoxicity to phenotypic measurements that include staining of cell surface proteins with fluorescently tagged antibodies and sorting with flow cytometry, we carefully evaluate how epigenetic targets change T cells. Only epigenetic perturbations that lead to significant improvements in T cell functionality over untreated control groups in culture are selected for mouse studies. This requirement not only leads to fewer mice required throughout a project, but also drives faster discoveries.

Why were they not suitable?

Cultured 3D tumor models, or multicellular spheroids are still years away from replacing mouse tumor models. Tumors grown in culture lack the vasculature that develops in animal models (even in the presence of growth factors) and therefore rely on diffusion for oxygen and nutrients. A huge drawback of this kind of growth is the necrotic or dead internal tumor microenvironment that invariably develops.
Other methods for improving on this include co-culturing cancer cells with endothelial and fibroblast cells for more accurate tumor heterogeneity as well as drug induced blood vessel growth, but this approach is still a long way from replacing animal tumor models. Additionally, functional immune systems are required for much of our immunotherapy research. We manipulate the host response in mice and in culture to study how the antitumor response can be modulated. This requires recapitulating the complex interactions between the host and tumor, which is currently not possible outside of 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?

This estimate is based on previous data that we have obtained and published using these same mouse models. Additionally, we have consulted experienced colleagues for additional expertise throughout our experimental planning. Our research plan is centered on discovering epigenetic-based therapies that demonstrate large improvements in antitumor immunotherapy efficacy (or large effect sizes) over untreated or unmodified controls. Only T cell modifications driving significant functionality improvements (quantified through several assays) are selected for small scale pilot studies usually in groups of five

mice. Successful epigenetic candidates must demonstrate a large effect size or ≥ 2-fold reduction in tumor volume over control groups to be studied further in larger groups of 7-10 mice.

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

Through experimental refinement and consulting experts in the field, we have been able to effectively reduce our mouse numbers. We have extensively reviewed the literature on activating and expanding mouse T cells in culture and much of our previous and ongoing work is focused on optimizing and refining this process. Improving T cell expansion reduces the number of tumor-bearing donor mice required for our tumor-infiltrating lymphocyte adoptive cellular therapy (TIL-ACT) experiments and ultimately creates a more durable T cell product that is more effective at lower infusion numbers with a greater effect size requiring fewer recipient mice to obtain statistical significance. Additionally, small-scale pilot studies are used to measure efficacy and effect size and provide a cut-off threshold to progressing to studies with larger group sizes and additional experimental groups.

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

Our measures for reducing mouse numbers start with optimized T cell culture conditions. This was achieved through an extensive review of current published methods on the expansion of mouse T cells as well as consulting experts in the field. Optimal T cell expansion, largely dependent on an optimal combination of cytokines, has reduced our donor mice requirements by half. Our current method allows us to passage T cells in culture longer while preserving enhanced proliferation functionality. Improving on this method even more is a constant pursuit in our work and will lead to future reductions in mouse numbers.

Efforts at minimizing mouse numbers continue with our tiered approach. Our earliest experiments involve epigenetic screens and functionality assays of T cells previously expanded in culture to workable numbers from a limited number of donor mice. Only after demonstrating significant improvements over untreated controls are specific epigenetic targets selected for mouse studies. These initial studies are conducted in small groups of five mice, a number previously determined sufficient for revealing moderate to large effect sizes. This pilot attempts to validate the findings in obtained in culture and measure how improved functionality translates to enhanced antitumor T cell activity within the tumor microenvironment. Only modifications that demonstrate a ≥ 2-fold reduction in tumor volume over controls are then eligible for further studies with larger group sizes (n=7-10) if needed. This requirement ensures that only T cell modifications with demonstratable antitumor activity are used more broadly in larger mouse studies and therefore further reduces our mouse numbers.

Refinement

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

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

Every step of the protocol has been carefully reviewed to ensure that any pain and suffering is prevented and if unavoidable, that it is mild and transient. Our welfare refinement is achieved in part by performing some procedures on unconscious mice to help eliminate acute pain and suffering. This approach also improves experimental refinement by creating more controlled conditions for performing successful subcutaneous injections.

Another important aspect of refinement in our research is frequent welfare checks of all mice in a study. These checks increase in frequency from every 2-3 days after cancer cell injection to daily when measurable tumor growth is visible. These daily checks include brief visual and hands on evaluations of overall health status and the tumor region and are performed for the duration of visible tumor growth. Daily checks will become twice daily checks in mice with decreasing Body Condition Scores or tumors presenting with signs of elevated ulceration potential (large tumors presenting with multiple visible signs of reduction to skin condition e.g. elevated redness, peeling, scabbing). In general, any mouse presenting with signs of impending reductions in health and wellbeing are assessed carefully and monitored with appropriate frequency to ensure that any welfare issues are promptly addressed or the mouse is humanely killed.

The restraint and tail vein injection of T cells are performed on conscious mice while anaesthesia is optional for subcutaneous cancer cell injections. Based on previous experience, it was determined that the anaesthetic induced cardiac depression would make tail vein injections more difficult, potentially increasing injection attempts, and leading to higher numbers of untreated mice or increased tissue damage and prolonged mild pain. Most procedures will be performed on conscious mice to limit adverse effects from frequent anaesthesia.

Tail vein injections are most refined by placing the recipient mouse into a 37°C incubator box for up to 15 minutes to induce vasodilation prior to restraint and injection. This approach aids in both the identification of blood vessels and positioning of the needle into the vessel lumen leading to shorter restraint times and successful injections.

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

Ultimately, our goal is to discover and develop novel and effective immunotherapy approaches for treating cancer in human patients. With mouse models, our preclinical studies are more likely to translate to human cancer treatment than studies in a less sentient animal such as fish. Mouse models are widely used in cancer research due to their physiological and molecular similarities to humans and more recently, several cancer models have been genetically refined to better recapitulate human cancer development and heterogeneity. These characteristics and resources are absent with less sentient animals and therefore replacing mice with a lower species currently is not conducive to our research goals.

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

All mice will be monitored every 2-3 days post-tumor cell injection until measurable tumor appears then monitored daily for duration of measurable tumor mass to monitor for ulceration. These daily checks consist of very brief visual/hands on inspections to assess general welfare condition. Mice presenting with mild forms of redness, soreness, peeling and scabbing at tumor site will be monitored twice daily for potential ulceration (cratering or pitting at the tumor).
Ulceration is a humane endpoint.

All mice with measurable tumor mass will receive Body Condition Scoring (BCS) every 2-6 days to be performed concurrently with tumor measurements and weighing. Mice with visible reductions in health and wellbeing are monitored more frequently and a weight reduction of ~10% will be treated with moist palatable food at cage floor. Mice presenting with low body condition scores or weight loss >15% are humanely killed.

Non-aversive supportive mouse handling with cupped hands/tunnel handling in place of picking up by the tail will consistently be implemented during monitoring both to closely examine the health of mice and to habituate them to handling.

Mice may be anesthetized up to two times in a protocol. Both occurring before subcutaneous tumor cell injections (primary and optional rechallenge)

Mice receiving intravenous tail vein injections will be placed into a 37°C incubator box for up to 15 minutes to induce vasodilation prior to restraint and injection. Restraint will be < 5 minutes.

Single use needles are used for all injections

Mice that received TIL-ACT are monitored closely for autoimmune disease through frequent welfare checks and Body Condition Scoring. Mice with visible reductions in health and wellbeing are monitored more frequently and receive moist food.

Mice with tumor rejection following TIL-ACT treatment with transformed T cells containing inducible apoptotic switches receive a bioinert small molecule intraperitoneal injection every 2 days for 4 injections. During this time, daily welfare checks are performed with visual and hands on assessments and Body Condition Scores/weight are assessed to monitor for signs of cytokine release syndrome and other complications of elevated cell death. These daily checks continue for seven days after the final injection or longer if required.

Dry food will be provided on cage floor for mice unable to efficiently reach the food hopper due to large tumor burden and mice with visible reductions in health and wellbeing or low Body Condition Scores/ ~10% weight loss will receive moist food on cage floor.

Alpha Dry Bedding will be used in place of traditional Aspen Woodchips for tumor bearing mice to provide a softer and more absorbent environment for larger tumors and potential wound management.

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

To ensure that our mouse experiments are always conducted in the most refined way possible, we will communicate often with animal care staff, participate in workshops when they are offered and regularly consult the NC3Rs website for up-to-date references and

protocols that reflect current best practice of the 3Rs. Additionally, we will consult the online collection of best practice protocols offered through the 3Rs Knowledge Bank, when available.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The 3Rs training resources offered online through the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) will be reviewed by the project license holder every few months and shared with all personal license holders. In addition, every member of the group working under the project license will register for the NC3Rs monthly newsletter and attending workshops and webinars will be continually discussed and encouraged during group meetings. We will take a team approach to our animal research through open communication and collaboration with all animal care staff in order to achieve high quality research with uncompromised animal welfare.

36. Neuronal communication in the brain of mice

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

brain, cortex, synapse, neuron, tau

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

A central goal of neuroscience research is to understand how the brain processes external and internal stimuli to coordinate cognitive and behavioural processes. Ultimately, this understanding must relate behaviour to the activity patterns of neurons and their synaptic connections within key circuits of the brain. Elucidating how the activity of these circuits becomes abnormal is also crucial to understanding pathological situations such as neurodegeneration. This project deals with these quetions in an unusually direct way - by observing the activity of neurons and synapses in the brains of live mice as they process sensory stimuli (1-4). Optical methods such as multiphoton microscopy now provide the resolution required to image neuronal and synaptic activity in awake animals and we will use these methods to make a circuit-level analysis of sensory processing and neuropathologies such as Alzheimer’s Disease.

Our aim is to understand how nerve cells and their synaptic connections convey information (e.g visual or spatial) during health and disease.

Our key questions are:

What is the nature of the neural signals by which information is processed and transmitted in the visual system and hippocampus in awake behaving mice?

How is the transmission of neural signals at the synapse altered by changes in behavioral state of the animal?

How is the processing and transmission of neural signals altered in neurodegenerative disease 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?

This work is expected to have benefits in three broad areas:

1) By elucidating how different types of neuron respond to different types of visual stimuli, it will yield a greater understanding of how the brain executes vision.

2) By concentrating on imaging the activity of synaptic connections, it will reveal how these key neuronal compartments alter visual signals and how they alter the transfer of neuronal signals when the brain enters different states, such as switiching from "sleep' to "alert".

3) By studying how the operation of neurons and synapses is altered by the accumulation of proteins that are known to cause neurodegeneration, we hope to suggest novel therapeutic targets and strategies.

What outputs do you think you will see at the end of this project?

The main benefit of our research is to increase knowledge about brain function. We currently know little about how the brain works at a cellular level. We study this problem in the context of a key function of the brain - the processing of visual information. In partcular, we want to understand how the processing of a visual stimulus changes under different circumtances. This can occur when an animal changes it's behavioural state by becoming aroused, or when it habituates to a stimulus that has become familar or learns how a viual stimulus leads to a tasty food reward.

The goal of this project is to contribute the following milestones:

Understanding changes in visual processing assocated with arousal or learning

Understanding how these behavioural effects are mediated by the neural circuitry of the brain, including the retina and visual cortex

In addition to new knowledge about the brain, we will also contribute by developing techniques to learn about the brain. We will refine methods to record functional activity of single retinal ganglion cells (neurons that transmit information from the eye to the brain) over a long time period of weeks to months. We will also improve methods for observing the activity of the synaptic connections that transmit signals between neurons.

The knowledge we gain will be published in peer-reviewed articles. Manuscripts will also be published on a preprint repository so findings are available to public as soon as we deem them ready for publication. We will present findings at conferences and during invited talks.

Datasets of neural recordings and simultaneously recorded behaviour of animals will be published online, and code developed by our group to analyse the data will be made publicly available.

At the end, we will have a better understanding of the changes in viual processing that underly adjustements in visually-driven behaviour of mammals.

Who or what will benefit from these outputs, and how?

This work is expected to have benefits in three broad areas:

By elucidating how different types of neuron respond to different types of visual stimuli, it will yield a greater understanding of how the brain executes vision.

Short term (1-5 yrs): novel data sets collected showing how neuronal activity changes in an awake, behaving animal; alterations in these parameters occurring under different brain states (e.g. resting, aroused, locomoting); measurements of activation of noradrenergic or other projection neuron terminals concurrently with the above data sets. ·

Intermediate term (3-6 yrs): These data sets will be incorporated into models of how the visual cortex processes visual information under different brain states, contributing to worldwide efforts to understand the function of the cortex.

By concentrating on imaging the activity of synaptic connections, it will reveal how these key neuronal compartments alter visual signals and how their input-output relation is modulated under different brain states.

Short-term (1-5 years): novel data sets collected showing how synaptic activity contributes to signal processing in the cortex of an awake, behaving animal; how the strength of synaptic connections is altered under different brain states (e.g. resting, aroused, locomoting); how neuromodulatory inputs that the cortex receives from other brain areas alter synaptic function.

Intermediate term (3-6 yrs): These data sets will be incorporated into models of how cortical processing of sensory information is modulated under different behavioural states

Long term (>6 yrs): Improved understanding of the normal function of the sensory cortex.

By studying how the operation of neurons and synapses is altered by pathologies such as the deposition of tau fibrils, the project will determine the earliest functional deficits associated with neurodegeneration and suggest novel therapeutic targets and strategies.

Short term (1-5 yrs): We will have much improved understanding of how brain function is altered in mouse models of neurodegenerative diseases, concentrating on two key areas of the brain – the visual cortex and the hippocampus. The new data we will provide will be based on direct (real-time) observation of neural and synaptic activity in the awake, behaving, mouse. Specific changes in synaptic function will be related to changes in the function of the visual cortex (vision) and hippocampus (spatial memory).

Intermediate term (3-6 years): Continued collaboration with pharmaceutical companies to investigate compounds that might block the pathological effects of neurodegenerative disease models on the function of neural circuits in the visual cortex and other brain areas. Potential identification of novel therapeutic targets and strategies.

Long term(> 6 yrs): Increased understanding of disease processes underlying Alzheimer's and other neurodenerative diseases will allow improved therapeutic strategies to combat these disorders.

How will you look to maximise the outputs of this work?

We will maximise outputs by dissemination of new knowledge at meetings and conferences, by publication of results in scientific journals and by collaborating with other research groups to investigate similar questions. We will also collaborate with colleagues at our establishment who study the visual system or particular behaviours in mice and promote the publications of our articles and datasets on social media channels like Twitter and via the media office of our establishment.

Species and numbers of animals expected to be used

- Mice: 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Brain architecture of mice and particularly the visuomotor system that we will study is very well conserved across mammals and is thus similar to that of humans. Our findings will thus have a high probability to generalise to other mammals and humans.

Mice use vision in their natural habitat and they can be trained on tasks that require vision. Using mice, we can study visual processing in the brain and how changes in this processing influence behavioural performance.

Many genetic tools exist for mice. These tools enable us to study specific cell types, and measure and manipulate neural activity.

Mice can be kept in animal facilities typically provided at a university setting under conditions that cater their well-being and health.

We will be using adult animals because we are studying fully developed brain function.
Typically, what will be done to an animal used in your project?

Almost all of our mice will be bred in the local animal facilities.

Mice that will be used in experiments (an estimated 20%) usually receive an implant attached to their skull so they can be head restraint. We will gain access to their brain by removing a small part of their skull (about 0.5 – 5 mm in diameter). In some mice, we will induce the genetic expression of certain molecules by injecting viral vectors into the brain

or the eye. We may also implant light guides (tens to hundreds of microns in diameter) to optically access deeper parts of the brain. These interventions are necessary to measure neural activity, identify certain cell types, or manipulate neural activity. These procedures are all performed under full anaesthesia and under aseptic conditions, and animals will be treated with analgesia during recovery from the surgery. Several surgeries may be necessary to accomplish our goals, for example if it is better for the animal's well-being to split one surgery into two or when genetic expression needs to be induced at certain time points after the animal has been trained, where training necessitates the head restraining implant. No animal will undergo more than 5 surgeries (each lasting more than 10 min).

Mice are then acclimated to head restraint and to being placed on a motorized treadmill (if used) over several days before we perform experiments to record neural activity. These experiments last at most 4 hours a day. Depending on the method of recording, the experiments continue for several days up to several months.

Some animals will be trained to perform a specific task, e.g. they have to choose one of two visual stimuli. To keep the mice motivated to learn and perform the task, their daily water intake will be restricted, and they will be rewarded with water when performing the task correctly. Animals are monitored every day for signs of dehydration, weight loss and abnormal behaviour, and their water intake will be adjusted accordingly.

At the end of the experiments, the animals will be culled or perfused for further histological processing of their brains.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will undergo surgery under general anaesthesia but are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. None of the surgical intervention, implants or viral injections is expected to lead to long-term harm.

Head restraint and placement on a motorized treadmill will cause stress to the animal but usually only during the first few sessions while the animal is acclimated. Once the animal is used to the head restraint, most of the time animals do not show signs of stress and behave normally, e.g. they run on a treadmill or they groom themselves.

In some experiments, in which neural activity is recorded, the insertion of the recording probe (a few microns in diameter) may cause temporary pain when the dura, i.e. the skin surrounding the brain, is penetrated.

Under water restriction, mice will most likely experience weight fluctuations and may show signs of dehydration. These effects will be monitored every day and provision of water will be increased as necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

About 95% of the animals (2,000 of 2,100) are expected to undergo Protocol 1 (Breeding) and will experience a severity category of mild.

20% of the animals (420 of 2,100) are expected to undergo Protocol 2 (Measurements and manipulation of neural activity during behaviour), and thus will experience a severity category of moderate.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We will study how the brain processes visual input during different behaviours, and how this visual processing impacts decision making and motor output. No artificial or non-living biological model system can replace the animals we use to perform our studies as we do not know the neural architecture and function that performs visual processing and controls behaviour.

To investigate how circuits in the brain contribute to the processing of information (e.g visual information in the visual cortex), we will need to work in vivo. This is the essence of our approach: to use the actual, unperturbed, neural circuit as far as possible. Cultures of neurons cannot see or navigate in space or carry out behavioural tasks that reflect the normal functions of the brain, and are therefore not an adequate substitute to understand how the retina or brain works.

As the function of neural circuits is profoundly altered by anaesthesia, many experiments will study unanaesthetised animals. This is also required to study processes such as motivation states. We will always seek to minimise animal use, however, and to maximise the information gleaned from every animal used.

Which non-animal alternatives did you consider for use in this project?

Parts of the brain (e.g. brain slices) and the eye (in particular the retina) can be removed from a dead animal and kept under conditions that allow nerve cells to stay functional. Such experiments are termed “in vitro” studies. These model systems can be used to study circuits of nerve cells and to some degree their function, e.g. which nerve cells are active together.

Artificial neural networks offer the opportunity to simulate biological neural networks.

Why were they not suitable?

In vitro models of populations of living nerve cells lack the input from the rest of the brain and they cannot be studied in relation to the animal’s behaviour, which is an essential goal of this project.

Regarding artificial neural networks, we have too little information about the biological networks in order to reconstruct them artificially. More importantly, we do not understand how nerve cells react and interact during natural 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?

Using statistical methods, we have determined how many nerve cells we need to record to answer each of our scientific questions and to draw robust conclusions from our results. Based on our previous experience, we have estimated how many nerve cells we will record from each animal. Both numbers together, i.e. the number of necessary nerve cells and the number of nerve cells recorded per animal, provide an estimate of the number of animals we will need to use for this project.

Several factors complicate the estimation of the numbers of animals though. First, there are uncertainties on the actual number of nerve cells we can record from each animal, as this depends on the quality of the preparation and the recording, as well as individual differences across animals.

Second, although we have planned a number of specific experiments that we want to perform, the exploratory nature of our research demands some degree of flexibility in the planning of experiments. In the case of highly unexpected results from one experiment, we may adjust subsequent experiments, which may lead to different numbers of animals needed. In the case of inconsistent results from performed experiments, we will perform further experiments on other animals if it is likely that these experiments will lead to consistent and robust results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental technology yields a large number of nerve cells that we are able to record in each animal: (1) we use state-of-the-art technology that allows simultaneous recording of hundreds to thousands of nerve cells; (2) we can perform these recording sessions several times on the same animal, so that every session yields another population of recorded nerve cells.

Our longitudinal experiments on awake animals last from several days to a few months. To gather the largest possible amount of data from each animal, and thus reducing the total amount of animals, we need to pay particular attention to the health and well-being of each animal. Please, see Refinement for details on this.
Our measures to control animal behaviour leads to shorter experimental sessions and reduces the number of animals. To investigate the influence of running on neural activity, we are introducing motorized treadmills to control when the animal is still or running at a certain speed, instead of relying on the animal to spontaneously sit still or run. As we need

to collect enough data in each condition, the control of running minimizes the necessary time needed for data collection.

When we are investigating functional differences between specific types of nerve cells, we will use several methods of targeting several types within the same animal. For example, we can use a transgenic animal to target one genetically defined cell type. At the same time, we can define other cell types based on the anatomical connection to other brain areas by injecting tracers into several brain areas.

We will constantly review the methods we are employing by following published literature or receiving advice from other experienced researchers in order to increase the effectiveness of our methods. This will reduce the number of failures, for example in targeting specific cell types, and will increase the success rate of experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

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.

We will collect multiple data from each animal, thus minimising the number of animals required. For example, in vivo imaging data of neuronal and synaptic activity will be collected from many hundreds of neurons in the one animal, from several regions of the cortex or hippocampus. The numbers of animals to be tested will be the minimum number required to obtain statistically reliable results, based on previous experience in the laboratory, and from published findings. Where appropriate, power calculations will be used to estimate the appropriate numbers of animals on the basis of expected variability, and anticipated effect sizes. Where possible, we will use within-subject comparisons to increase the statistical power of the experiments, and to minimise the numbers of animals that are used.

The tasks that are described here produce quantitative data, which is, in general, suitable for standard parametric statistical analyses, though non-parametric analyses will be used where data does not meet the assumptions (e.g. homogeneity of variance) required for parametric analysis. We shall use randomised block designs, where possible, to eliminate non-experimental sources of error. We have considerable experience of the use of statistical testing so that most designs will not require additional advice, but if necessary we can consult a number of statistics experts in the School of Psychology.

Cryopreservation will be used to preserve important lines and remove the necessity to hold stock for extended periods. On occasion we may need to ensure that our protocols are optimised and this may require the re-implantation of un-manipulated oocytes, embryos or blastocysts. Cryopreservation of embryos will be used for long-term storage of genetically altered mouse lines and pedigree lines with in vivo viability assessed to ensure that lines can be re-established successfully. Rederivation will be undertaken should the health status of the animals be compromised in a way that would significantly affect the welfare of the animals or where the experimental results might be altered unduly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In our experiments, we will use purpose bred mice, some of which are genetically altered so we can distinguish and target specific types of nerve cells. In comparison to many other mammals, particularly larger animals, mice can be kept in the laboratory under conditions that cater their health and wellbeing very well. The genetic modifications in the mice we are using are not expected to lead to pain or any harm for the mice. All animals will be co-housed with litter mates as mice are social animals. We will only make exceptions to this rule if the animal's health is impacted, e.g. when animals get injured by other cage mates or when animals are too vulnerable due to surgical implants or sutures.

We will perform surgeries to access parts of the animal's brain and to implant a plate that allows us to head restrain the animal. We always use appropriate anaesthetic and analgesic regimes for pain relief during surgery, and surgeries are performed under aseptical conditions. The implant weighs less than 1 g and does not affect behaviour of the animal in a negative way.

We will head restrain the animal during our experiments so we can reliably measure the activity of many neurons. At the same time, we will control the animal's sensory input, e.g. via visual displays, and its running speed (when placed on a motorized treadmill). We will acclimatize the animal being head restrained step by step, starting by simply handling the animal and then restraining it just for a few minutes. After a few days, the animal is getting used to the procedure and does not show signs of stress anymore.

To record and manipulate brain activity we will use methods of two-photon imaging, electrophysiology, opto- and chemogenetics. Two-photon imaging can be performed on awake animals on a daily basis for weeks and months without any invasive procedures in addition to the initial surgery. The same is true for the manipulation of activity via optogenetics. Manipulation of activity via chemogenetics requires the injection of specific drugs that then only act on previously targeted neurons that express receptors for these drugs. Electrophysiology necessitates the insertion of a probe into the brain to record electrical activity of the neurons. The recording probes we are using are only a few microns in diameter, much thinner than a human hair. The animal might feel mild pain when the probe penetrates the membrane surrounding the brain (the dura), but does not feel pain as the probe penetrates the brain itself as there are no pain receptors in the brain.

We will train some of our mice to perform a visual decision task where they have to choose the correct stimulus out of two presented stimuli. We do this to study how the brain processes visual input and it uses this information to make decisions. To keep mice motivated to learn and perform this task, we restrict their daily water intake as they are rewarded with water when they perform correctly. We monitor the animals every day for signs of dehydration and ill health and increase their water intake accordingly.

Our research depends on the mice being healthy, cooperative, and engaged in the tasks that we trained them to perform, so we have many reasons to avoid any suffering. The measures we take to avoid any unnecessary suffering work: the mice are cooperative and engaged.

Mice are the best experimental model for use in this project for a number of reasons. They share similar brain structures and functions with other mammals, including primates (but unlike non- mammalian vertebrates). Importantly for this project, a number of transgenic mouse lines are available, which will allow visualisation of specific cell types (e.g. Cre driver lines targeting different types of inhibitory interneuron), neuronal calcium reporters (e.g. GCaMP6/Thy1), reporters of synaptic activity (SyGCaMP or iGluSnFR) and manipulation of cellular function (e.g. APPSWE mice). Mice have been well-studied both in the fields of information processing within neural circuits and the disease models proposed here, so that these results will be readily integratable within the field and should prove more translatable to humans than studies using lower order vertebrates. Finally, the small size of mice means that more brain is accessible within the <1 mm imaging depth possible using the 2-photon imaging methods proposed here, than is possible in larger species.

Why can’t you use animals that are less sentient?

A major aim is to understand how cortical procesing of visual information is altered by changes in behavioral state of the animal. We decided to use mammals, in particular mice, rather than species of a different class that may be considered less sentient, because the brain structures we are studying (in particlar the cortex) are conserved within mammals including humans but are absent or show greater differences across animal classes such as fish or birds. We also cannot use aneasthetized animals because these do not produce behavioural responses modulated by vision. Anaesthesia also fundametally alters the function of the cortex and makes it abnormal.

The brain is still developing until early stages of adulthood. Our goal is study processes in a fully developed brain, and we therefore use adult animals. A further advantage of using adult animals is that they do not grow anymore; implants mounted on the head therefore do not need to be adjusted for changing sizes of the skull.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is monitored throughout the experiments, and animals are humanely killed where necessary.

Using a chronic cranial window for imaging involves surgery from which animals quickly recover, and then are stable for several months with usually no decrease in life expectancy or quality of life due to the surgery. After surgeries, the animals receive analgesic to minimize pain and are monitored closely for at least three days.
Animals are acclimatised to the head restraint for several days starting with a few minutes of restraint on the first day. Animals are taken off head restraint if they show high levels of stress, and acclimation is continued on a slower rate for these animals.

Animals under water restriction are closely monitored every day for changes in weight and signs of dehydration and illness. Water is given in higher amounts or ad libitum to restore full health.

Training of animals on decision tasks is performed step wise starting at an easy level and slowly introducing more difficult conditions. The time point of introducing more difficult conditions is based on the performance of each individual animal. In this way, the animals are learning the task in the shortest time possible.

Throughout the duration of this project, we will adapt all methods if better strategies become known and available.

What published best practice guidance will you follow to ensure experiments are conducted in the 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.

We regularly receive information from NC3Rs working groups that disseminate 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 stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have regular discussions with the Named Persons and animal technicians at Sussex to review current approaches and whether there are any new 3Rs opportunities.

We receive the NC3Rs e-newsletter that provides updates on 3Rs events and publications.

We will attend relevant NC3Rs events and workshops and participate in the NC3Rs working group on 'high-yield rodent behaviour', through which we share best practice in this area;

We will attend relevant 3Rs symposia at our institution.

37. The Evaluation of Veterinary Medicinal Products

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Evaluation, Efficacy Studies, Vaccine, Medicinal

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to generate data to support the safety, quality and efficacy of veterinary medicinal products in regulatory applications.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This is a service licence that aims to provide an ongoing service to customers such as pharmaceutical companies and research agencies. It aims to provide supportive data for the safety, efficacy and quality of vaccines and similar therapeutic biologicals.
What outputs do you think you will see at the end of this project?

The work carried out under this licence aids the development and licensing of new medicinal products and improves currently licensed products, thereby contributing to improving animal and human health, food production and the control of infectious diseases. There is also an economic benefit to the consumer and farmers through more efficient production. These products are fundamental in controlling disease and the spread and effects of infectious pathogens in animals and humans.

Who or what will benefit from these outputs, and how?

The data generated from these studies is used by pharmaceutical companies to support applications for Marketing Authorisations for their vaccines or other veterinary medicinal products. The data also informs companies on the design of field trials and even whether to proceed with candidate vaccines. Batch release testing allows companies to sell their products in compliance with manufacturing requirements. Ultimately the work therefore ensures products released onto the market are safe and efficacious.

Profits from commercial work are reinvested to fund the development of other tests and research posts. This benefits the organisation as a whole, and therefore ultimately the animal health work we are engaged in.

How will you look to maximise the outputs of this work?

This is a service licence with outputs linked to demand from industry.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals selected have been proven to be the best model for the proposed testing and are specified by regulatory text of the European Pharmacopoeia.

Typically, what will be done to an animal used in your project?

Protocol 1 Avian Viral Vaccines: Test for Extraneous Agents in Seed Lots

For each test item, ten chickens at 14 d/o will be inoculated with the equivalent of 100 doses of the vaccine via i/m and the equivalent of 10 doses of the vaccine via eye drop. Inoculations are repeated 14 days later. Five weeks after first inoculations the chickens are bled to provide serum samples for serological analysis and euthanised.

A control group of five chickens are reared to 14 d/o, bled at the time of first inoculations to provide baseline serum samples and euthanised.

What are the expected impacts and/or adverse effects for the animals during your project?

As the inocula are vaccine seed lots, no adverse effects are anticipated beyond some transient localised swelling or inflammation around the i/m injection site.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Chicken - mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The response to inocula such as pathogenic organisms and vaccines is a very complex process and one that cannot be replicated in vitro and it is not possible to generate definitive safety and efficacy data without the use of animals. The design of many studies for safety and efficacy are specified in regulatory guidelines and by the Competent Authorities and specify animals as the detection system of choice. However, should alternative tests that are acceptable to the Regulatory Agencies become available, such as PCRs for the detection of viruses and whole genome sequencing techniques, then these will be used to replace animal tests.

Which non-animal alternatives did you consider for use in this project?

None, the use of animals is a regulatory requirement for the proposed work.

Why were they not suitable?

N/A

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used is specified in the regulatory guidelines so there is limited scope for reducing the number of animals. The overall numbers proposed are those for testing ten test items.

Ongoing results are monitored and when possible the knowledge used to reduce the numbers of animals in future tests. A reduction in the overall number of animals may be possible by using only the most susceptible species or stage of development or sharing control groups. New methods that further reduce numbers will be used, for example better inoculation regimes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Scientific expertise of the organisation, input from customers, discussion with regulators and statistical rigor ensures the number of animals used is minimised. Possible reduction is discussed with the Sponsor and adjusted according to the aims of the studies.

Ongoing results are monitored and when possible the knowledge used to reduce the numbers of animals in future tests, such as sharing control groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

N/A

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model and age is specified in the relevant European Pharmacopoeia monograph. Careful consideration is given to possible refinements on a case-by-case basis. In addition, data from companies and their communications with the regulatory authorities and feedback directly from the competent authorities may allow tests to be refined.

Why can’t you use animals that are less sentient?
The animal model and age is regulated in the relevant European Pharmacopoeia monograph and therefore cannot be altered.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Ethical husbandry techniques will be applied, as will environmental enrichment.

All plans undergo review by the Animal Welfare and Ethical Review Body. All require close monitoring of all animals used in Studies.

As well as pre-start meetings involving the NVS, NACWO and animal care staff to ensure current knowledge is brought to bear, projects are followed up by a wash up meeting. All aspects are discussed, was the project a success, what went well and if there was anything that could be done better. If there are any suggestions for refining the procedure they will be considered and if appropriate, incorporated into the protocol.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

HO The Harm–Benefit Analysis Process

HO Guidance to ASPA

HO Code of practice

RSPCA Guidance on Welfare of rabbits and chickens LASA Guidelines on substance administration NC3Rs web site

Agency Procedure - AP007: Code of good practice for the use of animals in scientific studies

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Contact with the NVS, NACWO and NIO through various forums and use of the library function which can scan for relevant publications.

38. Novel interventions for poultry red mite

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

parasite, vaccine, poultry, mite

Animal types	Life stages
Domestic geese (Anser anser domesticus)	adult
Domestic fowl (Gallus gallus domesticus)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will progress novel interventions for the control of poultry red mites and involves development of laboratory colonies of the parasite as well as testing new methods to control the parasite in the laboratory and 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?

Infestation of laying hen houses with poultry red mites (Dermanyssus gallinae) is a major problem for the egg-producing industry worldwide and costs the poultry industry in excess of £250 million per year in the EU alone. Current treatment options are limited and often ineffective. Poultry red mites are blood feeding (hence their colour) external parasites that

build up on the cages of birds kept for longer periods of time such as those found in egg laying operations (up to 60 weeks). Heavy infestations cause anaemia, increased irritation and restlessness, feather-pecking and an increased incidence of cannibalism. These behaviours have a considerable negative impact on both welfare and productivity. Poultry red mites have also been implicated as carriers for a number of important avian diseases (including avian influenza) and have recently been recommended to be listed as an occupational hazard for poultry workers on the basis of their allergenicity. The poultry red mite problem is likely to increase due to resistance developing to main chemical treatments and consumer demand (and legislation) driving reduced use of chemical treatments and enriched environments for birds (which results also in increased habitats for the red mites). We aim to control infection with poultry red mites by exploiting the host immune response to these organisms to induce immunity against the pathogen and investigate whether this induced immunity can form an element of integrated control strategies.

What outputs do you think you will see at the end of this project?

At the end of the project we will have generated new information on host:parasite interactions, laboratory culture and control of poultry red mite. We will disseminate this information through open- access publications and presentations.

Who or what will benefit from these outputs, and how?

This project is expected to provide data to support the development of novel interventions to control poultry red mite. In the short term, protection and vaccine efficacy data, and data supporting the immune correlates of vaccine-induced protection, for example, will be generated. These data will be generated from experimental in vitro and in vivo protection trials and from field-scale trials for effective prototypes and will benefit researchers investigating the mode of action and efficacy of these vaccines. In the medium to long term, these data will form the basis of the rational development of a novel intervention in the form of advanced prototype vaccines and integrated approaches. Successful vaccines would be developed further in partnership with commercial vaccine manufacturers to produce registered seed stocks of vaccine antigen which will then be used in the safety and efficacy trials required for product registration. Several animal health companies have registered an interest in this area and this will be of benefit to them in the development of a novel market for animal health and welfare vaccines. In the long term, the outputs of a vaccine development project will therefore benefit the animals (laying hens) through improved welfare and freedom from parasitism; egg producers through increased productivity and reduced usage of chemical acaricides; vaccine manufacturers and distributors through the ability to supply novel products to a market which does not have any competition from other vaccines for poultry red mite; consumers through freedom from chemical residues in eggs. The work will also provide outputs which will underpin the fundamental scientific knowledge for scientists working on vaccines to control ectoparasites in production animals.

How will you look to maximise the outputs of this work?

The results of our investigations will be published in open access journals appropriate for subject matter, 3Rs and scientific impact. All manuscripts will follow the ARRIVE guidelines [Kilkenny et al., 2010. PLoS Biol 8(6): e1000412] to promote accurate reporting appropriate for 3Rs initiatives.

Following the key project milestones we will attend international conferences (e.g. the World Association for the Advancement of Veterinary Parasitology Conference and the International Symposium of Parasite Infections in Poultry) to communicate progress and to emphasise the focus on refinement and reduction in this project, highlighting crossovers in

disciplines. We will attend the British Society for Parasitology Annual Spring meeting each year to communicate these aspects. We will interact with scientists on the Interreg Europe project MITECONTROL and the networks which remain from the EU COST Action FA1404 “COREMI” which promotes sustainable control of the poultry red mite through co-operation and multidisciplinary networking between scientists and stakeholders from member states. Through these networks we will make our technologies and findings, and thus the benefits of refinement and reduction in animal use, available to EU scientists.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 1100
- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Poultry red mites are obligate parasites - they can only survive if fed the blood from birds. In this project we will use adult birds as they are able to mount good immune responses to any vaccines that we are testing and, when we need to take blood samples from them, we can minimise the numbers of procedures required to obtain sufficient volumes of blood.

Typically, what will be done to an animal used in your project?

Birds will typically be injected with vaccines and blood taken from them to monitor their immune responses. Small areas of feathers may be plucked from the birds' legs to allow access to monitor vaccine efficacy. Experiments may typically last between 10 and 20 weeks and birds would typically receive 2 injections of vaccine during that period and have 5 blood samples taken.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection may cause transient pain, though previous studies have indicated no or minimal reactions at the site of injection of red mite antigens. Vaccines will be prepared using sterile technique to avoid introduction of infection. Following feather plucking, mild transient discomfort may be caused. This will be mitigated by plucking small areas.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?
The expected severity for all procedures is expected to be mild, for all animals.

What will happen to animals at the end of this project?

- Killed
- Kept alive

- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Dermanyssus gallinae (poultry red mite) is an obligate but off-host parasite requiring to regularly feed on blood to complete its lifecycle, and has many similarities with ticks. The population of the poultry red mite is thus maintained through a complex interaction with the host.There is thus no practical alternative to the use of birds to test the efficacy of a vaccine to control this parasite. Searches for potential alternatives to the hen model using the current (24th August 2021) resources of the Fund for the Replacement of Animals in Medical Experiments (FRAME http://www.frame.org.uk) and the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs http://www.nc3rs.org.uk) have not yielded any useful alternative models. Laboratory-based blood feeding techniques are available to test the immediate effects of specific antibodies, which are incorporated into the blood meal. In addition, an on-hen feeding device is available to ensure accurate assessment of the efficacy of the vaccines prior to field trial. Although both these technologies still rely on the use of birds, the numbers of both birds and procedures required are very low compared with other methods of vaccine testing.

Which non-animal alternatives did you consider for use in this project?

None are currently available.

Why were they not suitable?

None are currently available.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on published data for the numbers of hens required for laboratory and field experiments to test a poultry red mite vaccine within the timescale of the licence and on the numbers of geese to be used as blood donors for parasite colony development in the same period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All animal studies have been, and will continue to be, planned in consultation with professional statisticians prior to submission to our local AWERB in order to provide

adequate group sizes for the most appropriate statistically robust analyses while minimising the number of experimental animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Analysis of efficacy and variability in our results will be used to inform a possible reduction in hen numbers in each treatment group in future experiments, with the proviso that reduction in numbers of experimental animals will have minimal impact of statistical significance of results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The poultry red mite is a specific problem for domesticated poultry which are, therefore, the only realistic choice of animal for these studies. The pathogens to be investigated in this project are obligate parasites and the model animals being used in this project are also the ultimate target species for any therapeutic emerging from the project. The models to be used include a mite challenge model where we test antibodies against mites in small-scale laboratory trials. While this requires a source of avian blood for the model, it reduces the numbers of animals required as a single blood sample can be used for testing antibodies (derived from eggs) against a range of antigens. To assess prototype vaccines in birds, we will also use a recently-published parasite feeding device in small scale trials involving only 5 birds per treatment group to remove sub-optimal vaccines. This technology was recently awarded the International 3Rs prize for it’s impact on both reduction and refinement of procedures.

Why can’t you use animals that are less sentient?

The parasite is specific for birds and terminal anaesthesia is not appropriate for procedures with a mild severity.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Monitoring post procedures will identify any areas which require refinement. For example, animals are closely monitored following vaccination and, if any of the compounds administered cause pain, appropriate analgesia will be administered

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Where appropriate, the World Association for the Advancement of Veterinary Parasitology guidelines for best practise will be followed https://www.waavp.org. In addition, the

ARRIVE guidelines 2.0 and PREPARE guidelines will be followed to allow the experiments to be performed in the most refined way and the publication of the data in the most appropriate form. (https://arriveguidelines.org/ https://norecopa.no/prepare).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The host institution holds numerous NC3R research grants and has regular contacts with this body. In addition, we have an active 3Rs committee, which provides advice to project and personal licence holders. Any relevant advances in the 3Rs will be implemented into any of the protocols where appropriate.

39. New therapeutics for inflammatory bowel conditions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Inflammation, Intestine, Therapeutics

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to facilitate drug discovery and development for the treatment of inflammatory bowel diseases (IBD) 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 incidence of inflammatory bowel conditions, such as Crohn's disease and Ulcerative Colitis (UC), is increasing in the developing as well as the developed world. This increase may be driven by changes to the types of bacteria that live in the gut, as a result of the increasing amounts of processed foods that we eat, and other environmental factors that we are exposed to, such as chemicals. These changes may trigger an unwanted

inflammatory response in the bowel. An individual's genetic make- up is known to influence their risk of developing long-lasting inflammation.

Inflammatory bowel diseases can be socially-debilitating due to the discomfort, pain and diarrhoea that they can cause; they can also get worse with time and can lead sufferers to develop other life- threatening diseases, such as bowel cancers. As the disease gets worse, patients require more powerful drugs to keep their disease under control. The drugs themselves have unwanted side-effects, such as immunosuppression, leaving patients vulnerable to infection. As a result of damage caused by the inflammation, the bowel may become narrow or stick to itself, or stick to the inside of the body wall (adhesions). Narrowing of the bowel may result in blockage and the adhesions can results in holes forming between different parts of the bowel, or between the bowel and the outside of the body; these issues require patients to have surgery to have the damaged bowel removed.

There is, therefore, an unmet clinical need for new treatments which are more effective, with less side effects.

What outputs do you think you will see at the end of this project?

The principal output from the project will be decisions on the potential for new drugs to treat inflammatory bowel diseases in humans. The identification of new drug targets may also be possible.

Where appropriate and permissible, data will be shared with the wider scientific community, through presentation at relevant scientific meetings and publication in peer-reviewed, open-access journals.

Who or what will benefit from these outputs, and how?

Primarily, the scientists who have developed new therapeutic strategies and agents for IBD will benefit from being able to make decisions about research priorities. Other researchers pursuing similar goals may benefit from what we find out, by making appropriate choices of experimental models or therapeutic approaches; for example, based on findings that may be presented or published by ourselves or our collaborators. Ultimately (although not within the lifetime of the project), it is hoped that people suffering with inflammatory bowel diseases will directly benefit from the output of the studies that we have been part of, with the availability of new treatments.

How will you look to maximise the outputs of this work?

Work output is dependent on using personnel who are trained and competent in all the experimental techniques required to deliver the aims of the project.

Planning and management of the studies undertaken are also critically important, particularly with regard to the choice of the correct experimental model and design of experiments, so that studies are fit for purpose.

Finally, data must be analysed and communicated effectively in order to ensure that their meaning is fully appreciated and that appropriate conclusions are drawn, and decisions made.

Underpinning all of the above, is the maintenance of animal welfare and the application of humane endpoints, in order to maximise the quality of the data obtained from a study.

Species and numbers of animals expected to be used

- Mice: 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 are using young adult mice in this project. This reflects the age at which first diagnosis of IBD is most common in humans. Adult mice will have a fully mature gut, including the presence of established populations of microorganisms within the gut. The use of animals allows us to investigate whether drugs can prevent disease and reverse established disease. These models provide essential information for making important decisions during the drug development process. Mice are the animal of lowest sentience in which we can perform these investigations.

Typically, what will be done to an animal used in your project?

In this project, mice will develop colitis (inflammation of the large bowel), that is similar to Crohn's disease and ulcerative colitis in humans. Induction of colitis is achieved in one of three ways: a chemical may be used to cause the inflammation; the mice may have been bred to have a mutation which results in them spontaneously-developing intestinal inflammation; finally, inflammation can be induced in mice which lack a fully functional immune system by injecting them with certain types of white blood cell from a normal, healthy mouse. The mice with colitis will then be treated drugs to see if they can prevent the development of the disease, or if they can reverse the disease process. These drugs may be administered by injection or given by mouth; they can also be administered in the animals' food and drinking water. Sometimes it may be necessary to perform small studies so that we can gain better knowledge of how and when we should give the drug to treat the disease, and what dose of the drug we should use. Blood may be sampled during the study. Mice can be administered compounds, in addition to the candidate therapeutics being tested, in order to measure changes associated with inflammation; subsequently, these compounds may be measured in blood samples. At the end of the study, mice will be humanely killed and tissues and blood taken for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that most mice will develop diarrhoea (soft and unformed stool) to some extent, unless they are receiving a drug that prevents the disease. The diarrhoea may be associated with weight loss, or reduced weight gain relative to mice that do not have colitis. The diarrhoea can make the mice dehydrated; the chemicals used to induce the colitis may result in some blood being observed in the stool. It is expected that there will be some observable decrease in the overall body condition of the mouse. The mice can experience some discomfort because of the colitis and this may show in the way they move around. The mice can be less inclined to stand or climb to feed from their food

hopper, so food intake may be reduced. Very rarely, there is the risk that a mouse may develop a non-resolving rectal prolapse. Mice may be expected to show mild clinical symptoms for a few days to a couple of weeks in some studies. The implementation of humane endpoints will limit the duration of more severe symptoms.

During the study, repeat injections may cause local irritation at the site. Mice may be restrained for the purpose of blood sampling from the tail vein, which may cause transient distress. Minor pain/discomfort may be experienced when blood is sampled. Very rarely sites of injection/sampling may become inflamed or infected. Discomfort from these procedures should be transitory and last for less than a minute

Some strains of mice used are immunodeficient and so are more prone to developing infection, such as inner ear infection, although this is rare. Cells from a mouse with a normal immune system can be used to induce intestinal inflammation in the immunodeficient mice; very rarely, these cells may cause inflammation in another organ, such as the lungs. Symptoms of these adverse effects will be recognised in routine welfare checks and limited by the application of humane endpoints.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

mild or less: 20 % moderate: 80 %

severe: not expected

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 gastrointestinal tract is perhaps the most complex organ system in the body. It must provide a physical barrier and is responsible for processing food, absorbing nutrients and excreting waste – all of which are continuously trafficked through the gut. It can be regarded as the largest organ of the immune system and is a first line of defence for the body, against invasive microorganisms. It contains more than 100 million nerve cells. It is home to several hundred species of microorganism, of which not all have been identified; in addition, not all can be grown in a laboratory. Gut health depends on the complex interaction between the cells that line the gut (epithelial cells), the immune cells in the gut and the bacteria (the gut flora). Modelling the complex interactions between the intestine and its immune system and the microorganisms that live in the gut is very difficult; cell culture models are only able to partly recapitulate this. The mouse models of inflammatory bowel disease demonstrate similar features to the disease in humans, such as the dependence on gut bacteria, the types of immune cells involved and the response to a

range of therapeutic agents. The animal models of colitis can also be predictive of drug action in humans; we have observed this with respect to a range of new drugs that have recently been licenced for treatment of IBD in humans.

Which non-animal alternatives did you consider for use in this project?

Three dimensional cell culture models using human cells are currently being developed. Initially, the complexity of these models will be low, allowing only a direct assessment of how the intestinal epithelial cells (the cells that line the intestine, forming the physical barrier between the inside and the outside of the body) repair following injury and how experimental drugs can modulate the repair mechanism. Subsequently, it is hoped to introduce other components to the cultures, such as immune cells and bacteria (or molecules derived from bacteria). Other laboratories (both academic and commercial) are also developing similar models; we are collaborating with one such laboratory.

These culture models allow us to look at how molecules directly affect the intestinal epithelium, the single layer of cells that line the gut. For example, they can be used to determine if molecules have effects that may be beneficial to the regeneration of this epithelial cell layer, such as changes to how fast the cells divide, changes to the proportions of cells with different functions within this layer, and how good this layer of cells is in forming an effective barrier to microorganisms. The 3D cell cultures can also be used for toxicity screening, which can help in eliminating candidate therapeutics from a pre-clinical development programme.

There will be an initially slow, but progressive accumulation of data that will allow us to compare the outcomes we observe in the cell culture models to those we see in the animal models. The ultimate goal will be to replace, where possible, the need for animal studies.

Why were they not suitable?

The three-dimensional cell culture models currently lack the complexity required to allow any definitive determination of the effectiveness of new drugs to treat IBD in humans. As mentioned previously, we are looking to improve these models and have a programme of cell culture model development in place.

The development of non-animal models depends on the availability of human donor material. The prospective acquisition of such material requires ethical approval and patient availability and so is a slow process (made slower by the current pandemic).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 quoted is based on animal usage over the term of the current licence. The figure quoted is for the whole five year period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When determining the number of treatment groups (and, therefore, animals) required for a study looking at the effectiveness of a new treatment for IBD, the following groups must be considered are:

Untreated (non-disease, baseline) controls, a reference control (a drug known to reduce disease, which is used as a benchmark for the Test drug or drugs) and a relevant placebo control for the reference (e.g. an inactive molecule with a similar structure, or the medium in which the reference drug is formulated).

The remaining groups in the study will be for the Test drug and its placebo control. Careful consideration is given to the range of different doses of drug that may be used in order to reduce the number of treatment groups and, therefore, the number of animals. The range of drug concentrations used is often based on previous studies using the drug, either in another animal disease model or in cell culture experiments; this can also be based on results from studies using similar molecules. These studies may have been conducted by ourselves or others.

The size of the effect we are looking for in response to a drug will influence the number of animals that we use. This has to be realistic and can be based on previous work using similar drugs. The smaller the improvement in disease that we want to detect, then the more animals that need to be used. We commonly set a threshold for being able to detect a 25 % reduction in disease with reasonable certainty.

Testing more than one drug in a study is a good way of reducing the overall number of animals used. In this way, we can minimise the number of mice used for control groups, particularly if the drugs are administered in the same way and at the same frequency.

We continuously review the data that we obtain from our studies in order to monitor how our models and or reference drugs and controls are performing. These data reviewed by independentstatistical experts in order to inform us of the correct number of animals to place in treatment groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We may perform small pilot studies in order to measure the concentration of drug that is achieved in the blood and in the intestinal tissue following its administration; this will be done with small numbers of mice per group (five or less), with one group per concentration of drug. Such studies can inform us of the most suitable concentrations to use in a bigger study to look at the effect of the drug on disease. The range of concentrations tested will be within the toxicity limits of drug; this will have been determined by previous work by other laboratories, based on animal studies and/or cell culture experiments.

We always seek to maximise the amount of information that we can obtain from each study. This is achieved by collecting clinical observations during the study and collecting as many tissue samples as possible, for analysis at the end of the study. We have available a wide range of analytical platforms to support our studies and give us access to multiple readouts that can inform us about different aspects of a drug's ability to suppress intestinal inflammation. In collecting as much data as possible from each study, we minimise the risk of having to run another study in order to gain further knowledge.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse models of inflammatory bowel disease. These models are well-characterised in terms of their biology and the onset of symptoms can be predicted and so a suitable frequency of monitoring animals for welfare purposes can be established and incorporated into study protocols. This also allows us to make informed decisions about when it is appropriate to end a study, according to the question we are trying to answer (by running the study). Humane endpoints are employed. All mice are humanely killed at the end of each protocol.

Why can’t you use animals that are less sentient?

Immature animals cannot be used as the intestine will not be fully mature and the population of microorganisms found in the intestine is also likely to be immature. These factors have a direct influence on immune responses in the intestine. The Zebrafish can be a useful model for studying genetic influences of intestinal inflammation, but testing candidate therapeutics can be challenging in such an organism and the skills and tools required to perform some of the analyses that are required are limiting. Our ultimate aim is to replace animal models where possible, so it is our opinion that our resources are best focused on this aim rather than establishing and validating another animal model. Mouse models are also the best-characterised and well-established models of IBD and employed by key opinion leaders in this field of research.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are monitored at least once daily and clinical observations are recorded. Data are reviewed daily in order to assess if any intervention is required. There are defined points in studies when mitigation such as mash food and increased frequency of observations are introduced. Appropriate guidelines will be followed with regard to best current practice for blood sampling and administration of substances to the mice.

During the project, we will operate a process of continuous review to ensure that the models we run are fit-for-purpose. This involves looking at all the data that we collect and seeing how it may change with time. We can then make appropriate changes to ensure that disease is not too severe, or that models run no longer than they have to.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will perform procedures according to our own standard operating procedures. We will also follow guidelines issues by the Joint Working Group for Refinement. *Lab Animals* (2001) doi: 0.1258/0023677011911345 and guidance from the NC3Rs, as appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about the 3Rs advances through attendance at lectures and seminars run locally, as part of a continuing professional development programme; through the 3Rs website; by keeping up with the latest scientific publications in the field.

40. Vaccination and Antibodies for Disease Protection

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccines, Antibodies, Immunology, Disease Prevention, Therapeutics

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop and optimise vaccines or antibody-based interventions against 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?

Vaccines are the pre-eminent cost-effective tool for reducing the burden of infectious disease, and it is the immune responses (especially antibodies) they induce that underpin their success. Indeed, in the past century, widespread vaccination campaigns led to the eradication of smallpox in humans and rinderpest in cattle, with polio also now on the brink; whilst most recently, the global success with new vaccines against Covid-19 has provided a route out of the pandemic. However, despite these great achievements, many difficult diseases remain against which highly effective antibody-mediated immunity fails to arise or for which effective vaccines have simply not been developed. In addition to the

known ‘big killers’ (malaria, tuberculosis and HIV), emergence and re-emergence of outbreak pathogens has become a serious threat to human health and the global economy, as exemplified by the SARS-CoV-2 pandemic and the previous 2015 Ebola virus epidemic. New approaches are also needed to tackle diseases in the ageing population.

In parallel to these advances in vaccines, antibodies have also made a huge impact in the field of biologics. Monoclonal antibodies (mAbs) have become essential treatments for inflammatory and autoimmune conditions, as well as cancer. With the more recent deployment of mAbs as treatment for Ebola virus and Covid-19, there is now renewed interest in the development of antibody-based biologics for other challenging infectious diseases.

The development and optimisation of new vaccines and antibodies against disease targets is thus critical to on-going efforts to improve global public health.

What outputs do you think you will see at the end of this project?

The below outputs are expected to arise by the end of this project:

- At least 10 peer-reviewed open-access scientific publications.
- At least 15 data presentations at scientific meetings or national / international conferences.
- At least 2 patent filings on arising intellectual property (IP).
- New information on vaccination strategies to achieve better immunity, prophylaxis or therapy for human malaria.
- New vaccine candidates and/or antibody biologics will be defined for human malaria that are ready to progress to clinical development.

Who or what will benefit from these outputs, and how?

- This project will mainly focus on the development of new vaccine and antibody candidates against the human malaria parasite. However, the technologies and approaches being used can be easily applied to other disease-causing pathogens, including viruses and bacteria, of humans and animals. We have a primary research interest in malaria parasites and vaccines, but also contribute our expertise to a number of other projects related to the prevention of human diseases such as Ebola, Covid-19, thalassaemia.

- We also work on vaccine platforms, i.e. new technologies that can be used to deliver vaccines. Typically we innovate in this area for malaria, because it is a very challenging disease target. However, often, the new vaccine platforms we develop can be easily transferred and used for other disease targets, e.g. pandemic viruses. In the medium/longer-term this means our outputs can find utility in a variety of different disease settings.

- The wider scientific community typically benefit from conference presentations (short-term) and publications (medium-term). New discoveries in malaria vaccine science are often broadly applicable across numerous disease areas (of humans and animals), meaning advances can be relatively quickly applied to other fields following presentation/publication of new knowledge.

- Arising IP for new malaria vaccines/biologics typically necessitates *in vivo* animal data. New patent filings bring benefit to the economy through licensing deals to companies

(can be short, medium or long-term dependent on the nature of the IP).

- New information on vaccination strategies and new vaccine/antibody candidates bring benefits to science and the economy in the short/medium term, as future clinical development projects are funded and undertaken. The ultimate beneficiaries of new vaccines/antibodies in the long-term are the public (human health) and/or animals (veterinary health) when new products are licensed to prevent and/or treat disease. The ultimate manufacturer of the biomedical product would also benefit financially and contribute to the long-term growth of the economy. In the case of malaria vaccines/antibodies, the ultimate beneficiaries of these products in the long-term are those individuals living in malaria-endemic countries (including those in Africa, Asia and South America).

How will you look to maximise the outputs of this work?

- We disseminate arising new knowledge by regular presentations at a wide variety of meetings/conferences (for scientists or relevant stakeholders / funders / health policymakers). We also publish arising data in peer-reviewed open-access scientific journals. We include all relevant data, regardless of whether the outcome would be deemed "successful or unsuccessful" in terms of vaccine improvement/development.

- We collaborate widely with numerous academic research groups and/or industry (biotech and pharma) at local, national and international level. Collaboration enables knowledge transfer andsharing of best practice.

- We participate in numerous national and international networks that are relevant to vaccine design, testing and development and enable sharing of new knowledge. New approaches/insights/breakthroughs in vaccines are typically transferrable to other disease targets in a short time frame.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use adult mice (from 6 weeks up to 15 months of age).

It is not possible to generate complex antibody responses outside of a functioning immune system in an animal. Mice represent the lowest order of species to enable us to meet the project's objectives.
The work is seeking to understand the immune response in an adult animal with a fully matured immune system, hence adult mice are used.

In some cases genetically modified mice will be required in order to understand the mechanisms by which we can develop improved vaccines and/or to enable specific types of antibody response to be induced. These genetically modified mice may also be bred for

use in this project.

Typically, what will be done to an animal used in your project?

The majority of mice will be vaccinated with a vaccine formulation intramuscularly on three separate occasions, typically at monthly intervals. Small blood samples will be taken from tail veins prior to each new vaccination to monitor antibody responses in the blood over time. Mice are typically killed 2-4 weeks after the last vaccination to measure systemic immune responses or to isolate antibodies from cells or serum. Most experiments are thus approximately 3-4 months' duration. If a longer experiment is required, mice will not be kept past 15 months of age.

Separately, genetically modified mice may also be bred for use in this project.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice may experience short-lived general malaise following vaccination, with mild symptoms persisting for 24 hours, just as occurs 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 animal type)?

Mice: 50% Sub-threshold, 45% Mild; 5% Moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune response to vaccination involves multiple, complex systems interacting in a physiological environment often involving antibodies, T cells and innate cells and therefore cannot be replicated in tissue culture. It is also not possible to generate complex antibody responses outside a functioning immune system in an animal.
Which non-animal alternatives did you consider for use in this project?
Wherever possible we replace the use of animals to study vaccines and/or generate antibodies.

We have developed a number of new *in vitro* assays that have allowed us to replace the need for animals to test the function of the antibodies that we induce by vaccination.

We use human cell samples from vaccine clinical trials to isolate antibodies *in vitro*.

We use *in silico* or *in vitro* methods to design/isolate antibody variants that could show improved potency.

Why were they not suitable?

In some cases it is simply not possible to answer scientific questions without vaccinating animals. However, we deploy a variety of state-of-the-art techniques that are highly complementary. As such, *in vivo* animal work provides a major avenue of scientific investigation, but it is one of many. We are thus able to use many other *in vitro*, *ex vivo* or *in silico* techniques to maximise knowledge gain first about vaccines and/or antibodies, and replace the need for animals wherever possible.

For example, it is not possible to fully optimise a new vaccine technology without confirming its ability to induce a physiological immune response. However, we use a variety of complementary scientific approaches to achieve this aim. Numerous analytical techniques are applied first *in vitro*, and only the most promising vaccine candidates are then assessed by *in vivo* immunisation.

Another example is the need for animal immunisation if no relevant human samples exist from which to isolate antibodies; these might include highly novel targets in a pathogen.

Similarly, *in silico* or *in vitro* methods rely on computational analysis or laboratory-based systems respectively which may not always be optimal, correct or able to out-perform nature's ability to improve or "mature" antibody potency by a functioning immune response.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

These numbers are based on prior experience and equate to 1800 mice per annum for 5 years. This includes:

- Breeding of 1200 mice per year (or 6000 in total for 5 years); and

- Experiments, whereby a single researcher may typically undertake an experiment with 30-36 mice; assuming 6 test groups of group size = 5-6. Three experiments of this typical size per year, would be 100 mice per researcher. Based on experience, with six research scientists working on the project, this would total 600 mice per year; 3000 in total for 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Throughout the project we will employ several strategies to reduce the number of animals used:

Prior to experimental work, statistical power calculations will be performed allowing use of

the smallest number of animals needed to provide satisfactory analysis of the data. We will also conduct our experiments so that we comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines) when we publish the results. We will also continuously evaluate and update our statistical approaches and group sizes, and make routine use of the NC3R's Experimental Design Assistant (www.nc3rs.org.uk/experimental-design-assistant-eda) and PREPARE guidelines (https://norecopa.no/prepare).

Past experience and/or new knowledge enables selection of experimental time-points that maximise the amount of data while using the minimum number of time-points.

Many experiments necessitate the inclusion of control groups to enable the comparison of new vaccines to a gold standard or most-promising vaccine to date. To minimise the repeated use of control groups, as many test conditions as possible will be included in an experiment. However the overall size of experiments will be limited as far as may be possible without compromising their scientific integrity. We will also aim to show statistical reproducibility of results with repeat control groups (e.g. those receiving a gold-standard vaccine), which would also allow for use of one control group across multiple future experiments.

Vaccine dosing experiments have provided us with reference data that allows us to select optimal antigen doses for investigation of immunogenicity. This has led to a reduction in the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible the data from each individual animal will be maximised through the collection of multiple tissue samples at endpoints and/or sequential sampling from the same animals across a time course, e.g. blood sampling during a vaccination time course.

The number of excess mice generated through breeding will be minimised by constant and careful monitoring of breeding programmes.

We are continuously refining/improving our antibody isolation methods, meaning fewer animals are needed to isolate rare/potent antibody clones of interest.

When testing new / exploratory vaccine technologies or approaches, pilot studies in a small number of mice will be performed to confirm viability of the approach (e.g. that an appropriate immune response is definitely observed and that there is no unexpected reactogenicity) prior to initiating larger experiments to test more variables.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

During the project we will vaccinate adult mice, and assess the immune responses. Blood samples may be taken to monitor responses over time. We continuously investigate ways to refine our techniques to minimise animal suffering.

Most vaccines used in humans cause transient local and/or systemic general malaise. This resolves within 24 hours. Some humans experience no side effects, whereas others experience some. It is not possible to predict. Consequently, it is necessary and inevitable that some animals may experience the same transient moderate general malaise when receiving clinically-relevant vaccine formulations.

Our previous experience ensures we can reduce the number of blood samples to only the key time- points post-vaccination. As the primary focus of our programme is clinical deployment, we primarily use vaccine delivery platforms that have been approved for use in humans and have minimal side-effects.

We also select routes of immunisation based on clinical relevance (e.g. intramuscular route will be the most commonly used route given that most human vaccines are delivered by this route, or alternative routes will be assessed if clinically relevant to the specific type of vaccine under study, e.g. the intranasal route would be used to assess delivery of a vaccine against a respiratory pathogen to the lungs).

Why can’t you use animals that are less sentient?

Mice represent the lowest order of species which will achieve the objectives of this project. Despite extensive efforts in the fields of immunology and vaccine development, there is no available alternative *in vitro* to using animals for the faithful induction of immune responses associated with vaccination. We have chosen adult mice for these studies since they are the most characterised species for detailed immunological analysis. Mice have proved to be excellent indicators of immunogenicity enabling the clear assessment of novel vaccines and vaccination regimens for improvements.

Mice also have the ability to raise highly effective and functional antibodies, many of which have ultimately led to licensed biologicals for human disease. Because of the numerous different immunological tools available, mice can also be used for detailed characterisation which is not possible in higher organisms and they also provide the unique opportunity to study the role of particular genes in the induction of an immune response by using genetically modified mice with defined gene modifications.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Routine vaccination requires use of needle and syringe as there is no alternative. Some needle-free technologies are in development by others, but remain highly specialised, in development, not easily accessed and not applicable to routine use. Chosen routes of administration are determined by clinical relevance and/or scientific experience. To minimise distress due to administration of substances, guidelines on volume limits are followed and the smallest needles possible will be used.

Blood sampling is performed from a superficial vessel in the mouse tail. Monitoring of immune responses in the blood is necessary, routine and clinically-relevant. From our past experience characterising the kinetics of the immune response, we are able to minimise the number of blood samples such that blood is taken at the most informative time points

post-vaccination depending on the type of immune response we are measuring.

When using a new type of vaccine, small pilot studies will be performed and mice will be checked at least once per day for at least 5 days and monitored for signs of general malaise.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All researchers will routinely discuss their techniques with the NVS and NACWO to get advice on refinement.

All researchers will regularly review websites with 3Rs information to monitor for potential refinements including:

www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will use local and national resources:

- All researchers will attend regular University/internal meetings where best practice on the 3Rs is shared.

- All researchers will regularly review websites with 3Rs information, including www.nc3rs.org.uk and https://science.rspca.org.uk.

41. Regulation of normal and malignant 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

Blood lineage specification, Blood stem cells, Leukaemia, T-cell therapy

Animal types	Life stages
Mice	embryo, juvenile, adult, pregnant, neonate
Xenopus laevis	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our main aim is to understand how blood cells are formed throughout life and the processes that convert normal cells into diseased cells with a focus on how aggressive leukaemia develops. This will guide efforts at understanding how to make blood cells to treat patients and to improve treatment of blood 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?

Blood cells are vital for oxygen and nutrient transport and to fight infections. When production of blood cells (haematopoiesis) is altered, leukaemia develops from rare cells

known as leukaemic stem cells (LSCs) that proliferate aberrantly and do not make normal blood cells. Understanding how blood cells are produced throughout life will help design methods to support to make blood for patients and identify novel therapeutic targets for treatment of blood malignancies and cancers and improve human health.

What outputs do you think you will see at the end of this project?

The specific outputs will be:

Refinement/establishment of new protocols for *in vitro* production of Haematopoietic Stem Cells (HSCs) from pluripotent stem cells or patient-specific cells.

Improvement of bone marrow transplantation technology and stem cell therapy through better ability to maintain, expand or direct the differentiation of HSCs.

Insight into the molecular and cellular processes by which normal blood stem/progenitor cells are transformed into leukaemic stem cells.

Development of less toxic and more effective rational combination therapies to eliminate leukaemic stem cells.

Development of sensitive tools to monitor residual leukaemic stem cells that could give rise to relapsed disease.

Who or what will benefit from these outputs, and how?

Short-term benefits: Mechanistic studies of blood production during embryogenesis will benefit developmental haematologists and, more broadly, developmental biologists as these will generate general principles. Molecular studies of production of normal adult cells and of leukaemia initiation and relapse will be of interest to the researchers studying both normal and malignant haematopoiesis as this will provide insights into fundamental oncogenic mechanisms. In leukaemia, identification of new therapeutic targets will be the first step towards development of new drugs. This will be of interest to the pharmaceutical industry.

Medium-term benefits: The ability to maintain, expand or direct the differentiation of HSCs will directly benefit clinical practices and patients requiring blood stem cell transplantations, by reducing transplant-related toxicity and death (currently, 5-10%). Newly designed therapies (for example human T cell therapies or combined therapies) will offer major improvements in the treatment and management of patients with leukaemia (acute myeloid and other types).

Long-term benefits: Patients with single gene blood disorders are currently treated with HSCs from donors (allo-HSC transplantation) with major issues due to lack of suitable donors and, when a donor is found, up to 40% mortality rate due to rejection of the graft. The ability to generate blood stem cells *in vitro* from patient-specific cells (auto-HSC transplantation) will benefit many haematology cell-based therapies. This would increase the survival rate currently achieved through allo-transplantation.

How will you look to maximise the outputs of this work?
We will disseminate our results at scientific conferences and in publications (choosing open access and general, rather than specialised, journals for broader dissemination), as well as through public engagement and engagement with patient groups. We will make

any new genetic tools and mouse models available to the scientific community through collaborations and deposition in appropriate public repositories for mouse strains. We will engage with clinical scientists and industry to translate therapeutic strategies towards clinical use.

Species and numbers of animals expected to be used

- Mice: 16300
- Xenopus laevis: 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.

Justification of species: Our studies ultimately aim at dissecting the regulatory mechanisms supporting production of human blood stem cells and at understanding how blood malignancies develop. Some of this work will be performed *in vivo* and we have chosen to work with mice, the lowest vertebrate species with similar physiological characteristics to humans. Mice are also the model of choice for genetic modifications of genes of interest for functional studies. In addition, the mouse is sufficiently similar to humans to allow human cells to be propagated *in vivo* using xenografting of immune-deficient animals. Lower vertebrate species such as frogs share key regulatory mechanisms with mammals and we also use this model organism for specific aspects of our studies.

Justification of life stages: Development of the blood lineage initiates at around day 7 to day 8 of mouse embryonic development (days E7-E8) and the first blood stem cells emerge at day E10. We will therefore focus our studies of developmental haematopoiesis around these stages and the corresponding stages of frog embryo development. In mice, the hematopoietic system reaches its adult state around weeks 4 to 5 after birth. We will use juvenile and adult mice to study normal haematopoiesis and how malignant haematopoiesis emerges and develops.

Typically, what will be done to an animal used in your project?

The majority of mice (60-70%) will be involved in breeding and maintenance only and will not experience any procedures. Moreover, a proportion of our work involves the study of embryonic stages before day 14 of embryonic development prior to protection under ASPA.

Experimental mice will undergo the following typical experiences:

Induction of gene expression in pregnant mouse females by substance administration (up to 3 times by intra-peritoneal injection), followed by humane killing and embryo harvesting.

Transplantation of blood cells into mouse tail vein or the femur following sub-lethal irradiation; administration of substances into the peritoneal cavity once a day for up to 28 days; blood sampling; bone marrow sampling (up to four times in total). Mice will be humanely killed.

Subcutaneous injection (up to 4) of primary human mesenchymal stromal cells by surgery; administration of human recombinant parathyroid hormone by subcutaneous injection daily for up to 28 days; transplantation of human blood cells transcutanouesly into the newly generated ossicles; administration of substances into the peritoneal cavity (once a day for up to 28 days) or into the ossicles (twice a day on a single day or once a day for up to 7 days); blood sampling; bone marrow sampling (up to four times in total). Mice will be humanely killed.

Subcutaneous injection of hormone into female Xenopus to induce super-ovulation; Humane killing.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts include transient discomfort or pain when substances are administered, when cells are injected, blood or bone marrow samples taken, and after surgery.

Development of hematological malignancy can be associated with fatigue due to anaemia. Rarely, the animal can experience more than moderate anaemia and show additional clinical signs, such as weight loss or not grooming appropriately. As soon as these signs appear, the experiment will be terminated.

Irradiation prior to bone marrow transplantation can cause transient mild anaemia for a few days.

Surgical procedures may rarely be associated with skin 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 animal type)?

Mouse:

Sub-threshold severity: 65%

Mild severity: 5%

Moderate severity: 30%

Xenopus

Mild severity: 100%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This programme investigates blood formation during early embryonic development, the adult bone marrow and leukaemia initiation and propagation in the marrow and throughout the blood. All of these biological processes happen in a highly complex environment. In development, blood formation occurs at a time of active cell migration through numerous cellular niches. In adults, the bone marrow is a highly complex tissue composed of many cell types. All of these processes occur in the setting of whole animal physiology, with metabolic changes and changing responses to multiple environmental challenges. Today, we do not have enough information to accurately model these processes *in vitro.* Moreover, *in vitro* studies do not mimic the metabolism, the complexity of engagement of the drug with different targets and the animal's and disease response to therapy. For obvious reasons, we cannot study the impact of untested therapies directly in patients. Therefore, *in vivo* models are required for our work.

Which non-animal alternatives did you consider for use in this project?

We have used and are still extensively using embryonic stem cells as well as haematopoietic cell lines than can grow using *in vitro* culturing methods as they allow some mechanistic insight and generate preliminary data.

Some aspects of blood cell differentiation and both normal and malignant stem cell maintenance can be mimicked *in vitro*, and we pursue these as methodologies when the question can be appropriately answered by *in vitro* techniques. In particular, we have considered the use of *in vitro* cultures for the maintenance and differentiation of haematopoietic stem cells, and for maintenance and screening of malignancy-propagating cells.

We are currently generating *in vivo* models of transplantation with more efficient engraftment of human samples by creating humanised bone marrow ossicles. Going forward, this will help define the physiological conditions necessary for the maintenance and proliferation of normal and malignant human haemopoietic cells, therefore leading to the development of *in vitro* assays in replacement of *in vivo* xeno-transplantation assays.

Why were they not suitable?

Despite the improvement of *in vitro* culture systems over the past decade, these models only partially recapitulate the 3D organisation of the embryo and provide information outside a physiological context, thus limiting the type of experiments that can be performed.

While *in vitro* colony forming assays can reveal progenitor lineage potentials, they do not read out fate.

We have been able to propagate some malignancy-propagating cells *ex vivo*; however, they rapidly lose their malignant properties, emphasising the need for a better understanding of the environment that sustains them. While we will continue to explore the methodologies mentioned above, they currently do not adequately replace *in vivo* experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experiments planned for this license are similar in nature to experiments we are currently performing, and animal numbers have been calculated based on the number of projects currently ongoing and planned, and our long-standing experience with this type of research, assuming that the number of PILs working on the new license will be similar.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Breeding strategies have been designed to minimise the number of non-experimental mice generated (e.g. by generating homozygous lines where possible without generating adverse phenotypes). Once work is completed, unused colonies are frozen down promptly. We source wild-type and common reporter mice from central colonies to avoid redundant breeding.

Transplantations: the number of animals used in experiments are calculated to ensure meaningful data can be obtained in experiments whilst keeping surplus animals to a minimum. We generated a database of available human samples in our biobank that have been transplanted into mice before, with information on engraftment for each sample analysed. This way researchers can select from those samples to address specific questions and don't have to test a number of new samples by transplantation into mice to find a suitable sample. We use our transplantation models to address different research questions in the same experiments. This reduces the total number of transplantation experiments that have to be performed. We routinely use intra-osseous injection (as opposed to tail vein injection) to minimize the number of injected cells that are lost in circulation and improve engraftment of small and rare populations of haemopoietic cells. This will result in a reduction of the total number of mice used in xenotransplantation experiments. Moreover, new mouse strains for transplantation of human cells have been established. These strains enable engraftment of a higher number of samples and higher levels of engraftment, thus reducing the number of mice needed to answer a specific question, as more transplanted mice will be suitable for analysis and the obtained cell numbers will be higher. We continuously look for novel mouse strains to improve transplantation efficiencies of human cells. Finally, the use of ossicles and scaffolds to generate an *in vivo* xenotransplantation model are being tested to achieve even more efficient engraftment of human samples, therefore overall reducing the total number of animals used.

Xenopus: We purchase wild type frogs from stock centres. As these are established lines, we only maintain minimal numbers. We have reduced the number of animals used by carrying out in vitro fertilisation enabling multiple females to be fertilised by a single male. We reduce the number of frogs used by buying testes rather than live animals.

Downstream experimental techniques have been optimised on low cell numbers to reduce the number of mice needed for adequate data collection.

We regularly consult Norecopa.no and NC3Rs websites for advice on experimental design. We will be conducting our experiments according to the ARRIVE guidelines when we prepare for publications.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding strategy: Genotyping of transgenic animal is done promptly to keep colony size to a minimum. When possible, we breed either wild-type mice, or heterozygous and homozygous mutant mice together to produce the genotype needed (for experimental and control mice) and, consequently, minimise the number of mice to genotype. The colonies are only expanded at times of experimental need. We will plan experiments within the lab, and where relevant across labs, share tissues and cells from mice.

We always set up pilot experiments when implementing new cellular/molecular assays in order to optimise our protocols on low cell numbers and to use the minimum number of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice: The use of bone marrow transplantation represents the most refined methodology for the identification and characterization of hematopoietic stem cells and haematological disease-propagating cells under physiological conditions. When required, irradiation is used for ablation of the endogenous hematopoietic cells. In many cases, this is a prerequisite for the transplanted cells to engraft. Irradiation is associated with transient discomfort. However, we will always use the least harmful methodology for transplantations, using sublethal or no irradiation where possible. When transplanting human cells, conditioning of haematopoietic cell transplant recipients with split radiation dosage to reduce morbidity and mortality to very low levels. We are also using immunodeficient mouse strains that allow for transplantation of human cells without irradiation conditioning. This reduces irradiation-related morbidity.

We avoid invasive techniques when administering drugs whenever possible by providing them in diet or drinking water rather than intraperitoneal injection. When required, invasive procedures are performed under general inhalation anaesthetic, and the animals are closely monitored after the procedure to ensure that normal function and behaviour is restored. Humane end points are refined during experiments to avoid unnecessary suffering of animals.

Xenopus: Haematopoietic development in Xenopus embryos shares many key regulatory pathways with higher vertebrates. Xenopus embryos are easily accessible and amenable to gene expression studies and genetic manipulations and are, therefore, an appropriate experimental model. To produce eggs for *in vitro* fertilisation, female frogs are injected with hormone by subcutaneous injection. This is the most refined and painless method to induce super-ovulation. To prevent the unnecessary use of animals, frogs that produce low quality eggs in two consecutive rounds of induction are humanely killed.

Why can't you use animals that are less sentient?

The mouse is the least sentient model with a haematopoietic system that sufficiently resembles the human haematopoietic system for the study of normal and malignant haematopoiesis to be relevant to human health.

The haematopoietic system only reaches its mature state 5 weeks after birth. To obtain results relevant to adult haematopoiesis, we therefore need to use adult mice. In addition, the vast majority of haematological cancers occur in adults, and their development occurs over an extended period of time, precluding the use of immature life stages and anaesthetised animals.**How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?**

Where required, local and systemic analgesia is administered immediately after a procedure. Mice are monitored once daily and up to 7 days, depending on the procedure, for signs of pain or adverse effects. Examples of post-operative care are provision of more analgesia for pain management, prophylactic antibiotics to prevent infections or palatable food at cage floor level in case of weight loss.

For mouse transplantation, we will use the least harmful methodology, using sublethal or no irradiation where possible, based on pilot experiments testing the feasibility of doing so.

For intravital imaging, we will seek the advice and training from researchers experienced in this technique (e.g. imaging facility of the MRC Weatherall Institute for Molecular Medicine or external experts) in order to ensure that no adverse effects result from terminal imaging.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgery will be performed following LASA aseptic surgery guidelines. We will use the NC3Rs Experimental Design Assistant (EDA) online tool or similar softwares to design experiments. We will conduct and report our experiments for publication according to ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All research staff working under this license will be encouraged to attend the annual institutional 3Rs day. We will consult the NC3R regional manager when necessary and relevant websites (NC3R, RSPCA Science group).

42. In situ molecular structures of the mammalian brain

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
Synapses, Memory, Alzheimer's disease, Advanced pathology, In situ structural biology

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of our research is to understand the structural basis of memory in the mammalian brain and how it is lost in Alzheimer's disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A key property of memory is that it is acquired within milliseconds yet retained for hours, days, months or even our entire lives. Importantly, long-term memory is not stored by on-going activity in the brain. Rather it is encoded by molecular and cellular structures. Therefore, investigating these molecular structures may give us insight into the mechanism of memory and how it is lost in neurodegenerative diseases of the nervous system.

Synapses are thought to be the molecular ‘microprocessors’ of the brain, mediate the correct ‘wiring’ of neuronal circuits, and mechanisms that are thought to be important for encoding memory.

My research exploits mouse genetic reagents and recent advances in cryo-electron microscopy to obtain in situ molecular structures of the mammalian brain, with a particular focus on synapses and neurodegenerative disease.

We use engineered mutant mice into which we have inserted genetic tags encoding fluorescent proteins. These tags are designed to label particular molecular structures within the brain and leave the healthy function of the mouse brain unimpaired. We also use fluorescent pharmacological labels where available. To investigate structures associated with neurodegenerative disease we use mouse models of Alzheimer’s disease. Of the existing models in vertebrate and invertebrate species, mouse models recapitulate the human diseases the closest.

Tissue samples collected from these labeled mice are frozen in vitrified state and imaged by cryo- fluorescent microscopy, wherein fluorescently tagged locations within the cells of the tissue are identified. We then image these samples on cryo-electron microscopy and map the locations. Next, we collect high-resolution images of the fluorescent locations as it is incrementally tilted in the microscope giving us different views. Finally, these views are computationally reconstructed to the in situ 3- dimensional structure. These experiments exploit advances in cryo-electron microscopy awarded the Nobel Prize in Chemistry 2017.

What outputs do you think you will see at the end of this project?

The major outputs of the research project:

Genetically altered mice

In situ structures of the mammalian brain.

Peer-reviewed scientific articles.

Who or what will benefit from these outputs, and how?

The mutant mice that we generate and use will provide a key resource for investigating molecular mechanisms of the mammalian brain and in the long term will contribute to devising mechanism-based therapies to treat diseases of the nervous system. These experiments will benefit the postgraduate students and postdoctoral research associates in training them in cutting-edge mouse genetic and in situ structural biology techniques. The in situ structures of brain that we acquire and insights gained from this information will be reported in peer-reviewed scientific articles benefiting the global neuroscience and Alzheimer's disease research community. The raw structural data and molecular models will be deposited in public databases, providing a resource for the scientific research community and pharmaceutical industry in perpetuity.

How will you look to maximise the outputs of this work?
I will maximise the outputs of this research by:

public engagement of the scientific discovery that we make.

I will promote the discoveries published from this project via press releases and social media announcements.

I will disseminate the research at international conferences, for example Gordon Research Conference 3DEM and AD/PD.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For several reasons, the project will use mice.

There is extensive knowledge of their neuroanatomy, physiology, and genome.

They have a similar genetic mark up to humans, sharing all but ~1% of each other's genes. The ability to manipulate the mouse genome permits the introduction of genetic alterations pertaining to human nervous disorders.

Typically, what will be done to an animal used in your project?

Mice will be housed and bred in a social environment whenever possible and provided with environmental enrichment such as chew sticks, fun tubes, nesting material, and platforms. Some mice will be left to age up to 15 months to allow development of the neurodegeneration which causes weight loss and progressive hind limb impairment. In some mice administer substances by a variety of routes, choosing the most suitable and least invasive route possible. Some of the routes will be non-invasive such as food or water. Other routes such as injection (under the skin, into the peritoneal cavity, into a vein, into a muscle), gavage, inhalation, intranasal will cause mice transient pain and/or transient stress. Some mice may receive surgical injections into defined areas of the brain: mice will be anaesthetised and a small hole will be drilled into the skull at the site of injection. Surgery mice will be given pain relief prior to recovery from anaesthesia and whenever necessary to alleviate pain as advised by the veterinarian. Following the administration of substances mice will be most often culled within 24 hours, whereas a minority of mice will be aged and to observe the possible development of neurodegeneration in brain and spinal cord, in which case these mice will experience weight loss and pain due to progressive hind limb impairment. At the end of experiments, mice will be humanely killed and samples collected for analysis. The mice may be killed under terminal anaesthesia by exsanguination to collect sufficient blood for protein.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of genetically altered and wiltype mice bred for use in procedures in this project will not experience adverse effects (subthreshold or mild severity). A small number of mice that carry a human mutation causing the accumulation of amyloid aggregates in

the spinal cord will experience hindlimb paralysis, mice do not suffer pain as this disease progresses (moderate severity). A small number of mice will experience transient pain that is not long-lasting through surgery with anaestetics or by the administration of substances to trigger neuronal activity (moderate severity).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

Mild: 70%

Moderate: 30%

Severe: 0%

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The brain structure of all mammals is similar and so the mouse is best suited for this work since, of all existing models, mouse models are the closest to the human diseases.

We developing our cryoCLEM approach to work with ES cell-derived neurons. However, so far, we have found these cells do not develop to form mature synapses as found in adult mouse brain.
Additionally, cells do not provide physiological conditions, the complex interactions amongst different cell types, nor develop the anatomical specializations found in tissue.

Existing animal models of common neurodegenerative diseases exhibit the essential features of the human diseases, a necessary prerequisite for the identification and evaluation of disease modifiers. As such, they are true models of at least some of the molecular and cellular features of the human diseases. At present, no valid alternative model exists.

Which non-animal alternatives did you consider for use in this project?

We are collaborating with colleagues generating neurons from induced pluripotent stem cells grown in the laboratory. This would enable the use of neurons derived from skin cells to be grown into nerve cells.

Why were they not suitable?

While this is ongoing research, currently we have been unable to produce cells and synapses comparable to those in the mature mammalian brain. The development of 3D organoids by colleagues in my field may also contribute to generating 'lab-grown' mini-brains. Currently, we do not have available protocols to develop these tissues to maturity, which is necessary for studying memory and modeling neurodegenerative disease. In the long term, this approach may be a viable alternative to using mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The following considerations have influenced our estimate of the number of mice used:

The number of different genetically altered mouse strains.

The length of the project provided by grant funding.

The number of mice needed for each experiment that will answer our research questions with a high degree of confidence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Mouse breeding will be carefully monitored to ensure that surplus animals are not generated. We will use the minimum number of animals needed to give a statistically significant result. A statistical expert will be consulted regarding experimental design and the most appropriate statistical analysis. Prior experiments and expertise with the animal model used will be considered when deciding on the number of animals needed for an experiment. Cryopreservation is being used routinely to preserve important mouse lines and to remove the need to breed mice only to maintain a given line.

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 performed pilots of our breeding strategy and established all the work-flows for obtaining high- quality in situ structures from mouse brains. Therefore, we have high confidence in the optimal number of mice needed for all our experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Existing animal models of common neurodegenerative diseases exhibit the essential features of the human diseases, a necessary prerequisite for the identification and evaluation of disease modifiers. As such, they are true models of at least some of the molecular and cellular features of the human diseases. At present, no valid alternative model exists. Only mice used in experimental studies will be allowed to develop neurodegeneration. Otherwise, only young mice will be kept.

Genotyping will be undertaken from ear biopsies except in those rare cases where more DNA is required when a tail biopsy will used.

When new lines are generated and bred for the first time, animal technicians will be specifically informed and the first litters carefully monitored. Any untoward phenotype will be discussed with the NACWO, veterinarian and if appropriate, the Home Office inspector.

The surgery will be carried out following "Guiding principles for preparing for and undertaking aseptic surgery" (2010) as closely as possible. Mice will be given analgesia prior to recovery from anaesthesia and whenever necessary to alleviate pain as advised by the veterinarian.

Why can’t you use animals that are less sentient?

We are investigating a key attribute of sentient animals, the structural basis of how memories are encoded in the mammalian brain. Therefore, less sentient animals at an immature life stage for example would prevent us from addressing this important fundamental question. Similarly, we are investigating the structural basis of Alzheimer's disease, in which patients' sentient attributes are eroded and lost, including memory. Thus, to model this disease in animals at a less sentient stage would not provide the best possible model of Alzheimer's disease. Alas, non-mice models, including invertibrates (eg. Drosophila fruit flies) do not have a nervous system sufficiently similar to that in mammals. Wherever possible we will use terminal anesthesia to perform experiments in animals that are less sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?
Good handling and injection technique will minimise any minor/brief distress associated with the systemic administration of test substances. Where multiple daily injections are given, the injection site will be changed to minimise pain and potential inflammation.

For surgical procedures, suitable anaesthesia and analgesia will be administered in consultation with the NVS and any sign of suffering will be discussed with the NVS for immediate advice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure experiments are conducted in the most refined way, best practice guidance in the following publications will be followed:

Handbook of Laboratory Animal Management and Welfare, Third Edition. September 2003. S. Wolfensohn & M. Lloyd. Blackwell Publishing Ltd. https://onlinelibrary.wiley.com/doi/book/10.1002/9780470751077

Guiding Principles for Behavioural Laboratory Animal Science. Edition One: November 2013. LASA. https://www.lasa.co.uk/wp-content/uploads/2018/05/LASA_BAP_BNA_ESSWAP_GP_Behavioural_LAS_Nov13.pdf

The Design and Statistical Analysis of Animal Experiments. March 2014. S.T. Bate & R.A. Clark. Cambridge University Press. https://doi.org/10.1017/CBO9781139344319

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed about new advances in 3Rs relevant to this project, I regularly perform literature searches, visit the NC3Rs website and receive NC3Rs email updates to ensure I am abreast of new research and procedures that will improve animal welfare. For all refinements, I will work with our Named Veterinary Surgeon (NVS), NACWOs, and academic colleagues to pilot improvements to animal welfare.

43. Investigation of the faecal phenotypes of ruminants - sheep model

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Proteomics, Metabolomics, Metagenomics, Faecal biomarkers, Animal nutrition

Animal types	Life stages
Sheep	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This work will apply advanced molecular biological techniques to identify proteins or other molecules that can be used to develop non-invasive tests to monitor the health of sheep, when they are fed on specific diets. Here we aim to use sheep as a model for ruminants in general.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 high proportion of British sheep and cattle is finished (fattened) in indoor fattening units

on high or *ad libitum* cereal diets. These diets are largely composed of barley, oats and maize, which contain somewhere in the range of 70 – 80% dry matter (DM) as rapidly fermentable starch. In general, high starch diets result in high rates of bodyweight gain and improved financial performance. However, as with most dietary inputs, there is an ideal rate at which cereals can be included, beyond which any increase results in reduced performance. Excessive starch supplementation results in increased loss in the faeces and contributes to digestive diseases, ruminal indigestion, which can result in a reduction in efficiency of production of meat of milk. There is an unmet need for farmers to be able to determine in real time whether their starch inputs are within an optimum band for maximal feed-use efficiency.

Existing guidelines for optimal feeding rates of starch in rations are not precise enough to account for variation in animal genetic and nutritional background, and there is wide variation in the amount and availability of starch in commonly used concentrate feeds. It is possible to measure faecal starch concentration in sheep and cattle, however the assay is cumbersome and expensive, and only a few laboratories undertake it. The characterisation of cattle and sheep faecal proteins has been limited. Work to date has looked at composite samples of faeces from animals on varied diets and at varying stages of production. It should be possible to develop a rapid, pen-side assay on faeces to determine if starch supplementation is within an optimal band. The faecal concentration of host (cattle or sheep) proteins appears to vary with diet, and we know that starch varies and can be measured. To identify the most effective markers of sufficiency of starch supplementation, further information is needed regarding the biochemical differences in faeces of animals fed differing diets when total intake, age and stage of production have been controlled.

The detailed characterisation of the biochemistry of faecal samples (ie the phenotype referred to in the title) using advanced molecular biology methods is intended to lead to easier, less invasive methods for assessing the health and production status of animals on diverse diets.

What outputs do you think you will see at the end of this project?

The new information that we hope to derive is the identification of proteins or metabolites in the faeces, which, alone or together, indicate the inflammatory, metabolic, and nutritional state of the animal.

It is expected that the study results will be published in peer reviewed publications. We aim to generate sufficient information for at least 3 publications.

Agricultural scientists, farm animal nutritionists and veterinarians will apply the methods to further explore the use of faecal markers in the study of animal nutrition.

If we can identify promising markers, then it is likely that our industry partner will invest in the development of a commercial tool for pen-side or laboratory use.

It is proposed that the techniques arising from these studies will lead to models of animal (nutritional) experimentation of higher welfare, and to improved dietary/rationing control, enabling real-time ration formulation and / or ration adjustment. More efficient rationing should result in reduced cost of production and lower environmental impact of production.

Who or what will benefit from these outputs, and how?

Farmers, agricultural nutritionists, animal scientists and veterinarians should all benefit

from this information.

The data collected in the course of this work may be used for the development of new products.

How will you look to maximise the outputs of this work?

Results of the study and new knowledge will be disseminated via publications printed and online. The results will be presented in national and international conferences. Our industry partner will promote the results of the study. If we are successful in identifying markers of efficiency, then we expect that this might be commercialisable IP of interest to our industry partner.

Species and numbers of animals expected to be used

- Sheep: 90

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The stage of interest to us is the late growing and finishing or fattening stage of life – sub-adult male animals that represent the majority type of animal that is fed on cereal in conventional agricultural practice.

Ultimately we aim to develop tools for application to all ruminants. We have selected sheep as a model in this study because of the reduced cost and increased ease of handling and experimentation at all stages. In animal nutrition science, sheep are a generally accepted, close model for cattle.

Typically, what will be done to an animal used in your project?

Sheep will be treated for internal parasites on arrival, by one-off administration of a de-worming drug (orally or by injection). On a subsequent single occasion, a small probe (ruminal pH bolus) will be administered orally to a proportion of sheep. Sheep will be subject to handling on several occasions (i.e., held by a trained and experienced stock-worker). On each occasion, when handled, a small blood sample and a small faecal sample will be obtained from each sheep, and the sheep will be weighed.
Sheep might be penned in individual pens for between 10 and 49 days. When penned individually, all sheep will have nose to nose contact with other sheep in the group. Sheep might wear a harness that collects all faecal output for a small number of 24-hour periods. All sheep will be offered differing diets and all diets will meet the sheep's nutritional requirements. However, some sheep will be offered diets without an initial transitioning period, and this might cause indigestion. These sheep will only be offered this diet for a period of 48 hours.

What are the expected impacts and/or adverse effects for the animals during your project?

It is possible that a small number of sheep (fewer than 15 individuals) will suffer from moderate signs of indigestion. This could include diarrhoea and loss of appetite. These signs are unlikely to persist for more than 48 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity: It is possible that 15 sheep out of a possible 90 sheep enrolled in the study would experience this severity

Mild severity: All other animals are expected to experience a mild severity.

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The purpose of the study is to assess the effect of starch-containing diets on the faecal phenotype (metabolome, proteome, and chemical profile) of sheep. This requires experimentation on sheep as the relevant target species. Consequently, alternatives to using ruminants have not been considered.

Which non-animal alternatives did you consider for use in this project?

In vitro ruminal fermentation is widely used to characterise digestive processes in sheep and cattle but these methods are not suitable for our study.

Why were they not suitable?

We are interested in faeces as our end sample, and the relationship between ruminal fluid and faecal composition has not been effectively characterised. We expect that much of the variation in patterns of biochemical composition that we will see are likely to be driven largely by distal gastrointestinal modifications, particularly in the caecum and colon.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 used reflects the different types of studies in the project overall. We need a relatively small number of sheep in the pilot study, in which we intend to apply most strongly contrasting dietary treatments. In the second sheep study, we need more sheep to deal with the increased number of treatments. It is possible that if the initial work suggests only small effects of dietary treatment on proteome and metabolome, we might need to reduce the number of treatments used for and increase the number ofanimals per treatment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Selection of numbers is based on what we consider to be the combined effects of the expected effect size and the experimental aims. The number of animals is the minimum number expected to provide sufficient empirical strength to disprove our hypotheses if they are not true.

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 preliminary data that suggests it is reasonable to expect measurable effects with the proposed scale. The initial experiments are the pilot studies for the work. They will later inform the design of further studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This work is directed at the development of proteomic based assays on faecal samples to quantify the efficiency of feeding starch-based concentrates to sheep. Two experimental methods have been developed to achieve this objective:

Feeding a high and low volume of starch-based concentrates to sheep without a transition period: As refinement, here we reduce the time of the challenge and use the smallest challenge expected to address the experimental requirements, although individually housed, all sheep will have direct nose-to-nose contact with one or two other sheep, all diets offered will be of a formulation that is commercially available, and we minimise handling stress by synchronously collecting samples, as infrequently as we can.

Feeding differing volumes of a starch-based concentrates to sheep with a transition period: As refinement, here we minimise handling stress by sample collection synchronously and as infrequently as we can, and all diets offered will be of a formulation that is commercially available.

Why can’t you use animals that are less sentient?

There is no relevant model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have selected interventions specifically to minimise welfare costs. We will apply a high level of monitoring of animals on the study at all times. We are using a short challenge period and a dosage that we expect will not result in any discomfort. All diets offered will be of a formulation that is commercially available. We minimise handling stress by synchronously collecting samples, as infrequently as we can, and where individually housed, all sheep will have direct nose-to-nose contact with one or two other sheep.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There is no specific guideline that is relevant for this study. The most useful document is the "Welfare of sheep: code of practice."

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The researchers involved subscribe to online notifications, receive institutional 3Rs information and are aware of the regular symposia that are organised on the subject. There is also collegial group membership of the institution’s welfare committee. Regular dialogue will ensure awareness of important developments.

44. Generation and breeding of genetically altered rodents

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words
Transgenic, Generation, Mouse, Breeding

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project licence are:

To generate mice with germ line genetic alterations and initiate their breeding in order to supply them to either the Establishment Breeding Maintenance Licence**,** research groups at our Establishment or to other establishments working in collaboration with us.

To rederive new lines imported from external sources with unknown health status for introduction into barrier accommodation and maintenance at a high health status in optimum environmental conditions.

To cryopreserve gametes/embryos to protect against genetic drift and in the event of unexpected disaster (eg. Infection in the animal unit). Archiving of lines will also avoid wastage from the need to maintain colonies by continuous breeding.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Generating new mouse lines will allow researchers to answer key questions related to understanding the function of genes and pathways in a wide variety of biological, physiological and pathological processes involved in cancers.

When mouse lines exist elsewhere, they will be imported into the Institute and will usually need to be rederived into the breeding unit. This allows researchers to share Genetically Altered Animals (GAAs) and for them to be used to answer different scientific questions.

Cryopreservation is a very important aspect when working with mouse strains. It not only serves to safely archive strains in case of disasters, but also slows down genetic drift, reduces the wastage of animals during continuous breeding and avoids the need to transport live animals, by shipping frozen material instead.

What outputs do you think you will see at the end of this project?

Newly generated, genetically modified mouse models will be embedded in projects from different research groups at the Establishment. The generation of new mouse strains is crucial for the development of research projects aiming at understanding the function of genes and pathways in a wide variety of biological, physiological and pathological processes (Khaled and Liu, 2014).

Once a particular mouse model is generated, it will constitute a fundamental tool that can be used by many research groups. Depending on the versatility of the genetic modification, many aspects of cancer research will benefit from the existence of these GAAs. For example, conditional gene activation and introduction of specific point mutations have been widely used to unravel gene function in different types of tumours.

Under the previous licence there has been a proven record of efficient generation and breeding of new mouse strains and this new PPL will continue and improve the provision of high quality animals to the researchers.

Khaled WT and Liu P. Cancer mouse models: Past, present and future. Seminars in Cell & Developmental Biology 27 (2014) 54–60.

Who or what will benefit from these outputs, and how?

This project aims to generate, breed and supply GAAs for the benefit of research groups at the Establishment and for those at other establishments working in collaboration with us.

There are around 14 groups at the Establishment using mouse models who will benefit immediately from the newly generated GAAs to fulfil their scientific questions.

How will you look to maximise the outputs of this work?

Once new mouse lines are generated they will be transferred to the researchers to perform all the required experiments. Within their research framework, all the results will be published accordingly.

Species and numbers of animals expected to be used

- Mice: 11,950

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Researchers have studied laboratory mice as models of human cancer for many years. Their use as cancer models has provided exceptional insight into the biology and genetics of this human disorder. There are standard protocols, methods and reagents used that have been optimised for manipulation of genes in this species and their acknowledged benefits for use.

Early embryonic stages will be use to introduce the genetic modifications required. Depending on the approach, 1-cell stage embryos or blastocysts will be used. Also, females around 3-4 weeks of age and 7-8 week of age will be used for superovulation protocols and as embryo recipients respectively. Vasectomies will be performed in males around 7-8 weeks of age.

Typically, what will be done to an animal used in your project?

GAAs will be created under this licence in order to understand further different processes associate with cancer. The various steps involved will be:

1: Injection of hormones to increase embryo production in female mice.
2: Female mice may have embryos implanted.
3: Vasectomy of male mice to allow them to be used to make females have phantom pregnancies and make them ready for receiving the test-tube generated embryos.

New mouse strains will be created by manipulation of the embryo using standard gene targeting methods. Each new strain will have a very well described, expected phenotype. However, animals will be monitored for unpredicted adverse effects. Surgical Procedures will be performed under anaesthesia and using pain relief and following aseptic methods to minimise risk of post-surgical complications. Anaesthesia will be carefully and regularly monitored to ensure that an adequate depth is maintained throughout any surgical procedure. Mice will be monitored regularly for their health status throughout all procedures. All procedures will be undertaken by trained, competent people.

What are the expected impacts and/or adverse effects for the animals during your project?

After surgeries animals will be closely monitored to assess their recovery. Analgesia will be provided before the surgery and after if required. Within these standard surgical procedures, it is estimated that any type discomfort will be transient.

When new mouse lines are generated, founders carrying genetic modification will be closely monitored for unexpected adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on Returns over the last four years we anticipate ~12% Unclassified; 67% Mild; 21% Moderate; 0.3% Severe. There have been no “Unclassified" since 2017 however.

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 purpose of the Licence is to generate transgenic mice for research purposes.

New technologies are improving the field of animal transgenesis and they will allow bespoke new mouse models to be applied in biomedical research. Although the very first steps in a scientific project will involve in vitro approaches, the final characterisations and implications will required the use of GAAs. The different animal models will integrate the complete range of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal biological processes.

Prior to creating a new strain, consideration will be given to the scientific evidence gathered from in vitro data. The breeding method and proportion of affected animals produced will also be considered and discussed with the researcher and the NVS as required. This information will be included in the Transgenic Mouse New Line Creation Justification Form (Appendix 1).

Which non-animal alternatives did you consider for use in this project?

Not applicable here given the Licence purpose.

Why were they not suitable?

Not applicable here given the Licence purpose.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 is estimated on the basis of yearly generation of 15 new mouse lines, perform 80 cryopreservations, importing 10 new mouse lines from external providers and perform 7 embryonic stem cell projects.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To avoid generation of already existing GAAs, all databases and cryopreservation banks will be interrogated. Examples of resources available include:

Jackson laboratory: https://www.jax.org/jax-mice-and-services/find-and-order-jax-mice International Mouse Strain Resource (IMSR): http://www.findmice.org/index International Mouse Phenotyping Consortium: http://www.mousephenotype.org
The genetic background of the strain used to generate a new GAA will be carefully considered and discussed with the user to avoid producing unwanted mice.

A full list will be maintained for all existing strains created and existing in CRUK MI. This comprehensive record will detail the nature of the mutation, any known adverse phenotypes, breeding and husbandry requirements. A full Mouse Passport will be maintained in house for all lines as a transgenic register providing husbandry and welfare information (Appendix 2).

Some reagents design to generate the genetic modification will be tested in vitro before using them in the embryos.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Cryopreservation of gametes/embryos and sperm will be carried as routine. This methodology will protect against genetic drift and in the event of unexpected disaster. Archiving of lines will avoid wastage from the need to maintain colonies by continuous breeding.

Super-ovulation using inhibin antiserum and equine chorionic gonadotropin increases the number of ovulated oocytes. This will result in enhanced production of embryos per mouse, therefore reducing the number of mice required to provide embryos for microinjection or cryopreservation of transgenic lines (Takeo et al., 2015).

Takeo T & Nakagata N. Superovulation using the combined administration of inhibin antiserum and equine chorionic gonadotropin increases the number of ovulated oocytes in C57Bl/6 female mice. PLoS One 10(5): e0128330 (2015).

The implementation of iGonad protocol aims to reduce the number of animals required to generate new mouse lines. The traditional procedure implies harvesting embryos from super- ovulated females (females group 1), injecting / electroporating them and afterwards, transferring them into foster mice (females group 2). In the iGonad protocol only one group of females will be used as embryos will be electroporated in vivo and the oviduct will be carefully returned to the body cavity.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Researchers have used laboratory mice as models of human cancer for many years. There are several reasons that make this rodent the best model to use: the mouse has a short reproductive cycle, large litter sizes, is easy to maintain and can be readily shipped from breeding facilities to research locations. The breeding methods we use will be undertaken under general anaesthesia where there is a risk of pain, suffering, distress or lasting harm.

The welfare of newly established and imported genetically altered animals will be assessed following the principle guidelines and recommendations provided by the report of the GAA welfare assessment working group (Wells et al., 2006).

Wells DJ, Playle LC, Enser WEJ, Flecknell PA, Gardiner MA, Holland J, Howard BR, Hubrecht R, Humphreys KR, Jackson IJ, Lane N, Maconochie M, Mason G, Morton DB, Raymond R, Robinson V, Smith JA and Watt N. Assessing the welfare of genetically altered mice. Laboratory Animals (2006) 40, 111–114.

Why can’t you use animals that are less sentient?

Researchers have studied laboratory mice as models of human cancer for many years. Their use as cancer models has provided exceptional insight into the biology and genetics of human disorders. The generation of new mouse strains is crucial for the development of research projects aiming at understanding the function of genes and pathways in a wide variety of biological, physiological and pathological processes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals will be given adequate analgesia and be closely monitored after surgical procedures. We use a document, that needs to be completed with daily observations, associated with each mouse that have undergone a procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Manipulating the Mouse Embryo. A laboratory Manual. CSH Laboratory press.
Reproductive Engineering Techniques in Mice. Naomi Nakagata.
Breeding and Colony Management Best Practices:

https://www.nc3rs.org.uk/sharing-archiving-genetically-altered-mice-opportunities-reduction-and- refinement

https://www.nc3rs.org.uk/breeding-and-colony-management

Wells DJ, Playle LC, Enser WEJ, Flecknell PA, Gardiner MA, Holland J, Howard BR, Hubrecht R, Humphreys KR, Jackson IJ, Lane N, Maconochie M, Mason G, Morton DB, Raymond R, Robinson V, Smith JA and Watt N. Assessing the welfare of genetically altered mice. Laboratory Animals (2006) 40, 111–114.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Every year our Establishment organises events where we can present and discuss new approaches related to the 3Rs. Also, I am a part of a working group in the NC3Rs that meet regularly to generate online resources about colony management and archiving.

45. Studies on immune regulation and tolerance

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Tolerance, Immune regulation, Transplantation, Autoimmunity, Cellular therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	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's the aim of this project?

The overall aim of this project is to examine the mechanisms that are responsible for immune tolerance, and to further develop existing therapies or identify new therapeutic targets that promote tolerance for pathologies such as autoimmunity and transplant rejection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 plays a central role in maintaining human health. When dysfunctional, the consequences can result in serious and life-threatening diseases. In autoimmunity, the

immune system is unable to maintain tolerance to 'self' and therefore can attack and destroy cells and tissues. Identifying methods to prevent this from happening has implications for a number of diseases including diabetes, inflammatory bowel disease, and psoriasis. In transplant rejection, the immune system is functioning normally and detecting the transplant as 'foreign', therefore initiating a destructive immune response against the transplant which can result in graft loss, organ failure, and patient death.

In both autoimmunity and transplant rejection, regulating and controlling immune responses is key. The immune system has inherent regulatory mechanisms that normally function to restrain its activity and prevent an overshoot of immune responses. Over the past decade or two, we have identified methods to harness these physiological mechanisms to control immune responses therapeutically, with some of these therapies successfully translated from animal models to human clinical trials. Specifically, we have identified and developed cellular therapies ("living drugs") which exploit the immune system's regulatory circuits and started to test these in humans. This project aims to continue this important work to further develop these therapies for eventual translation to clinical trials for patient benefit.

What outputs do you think you will see at the end of this project?

The main outputs of the project will be:

Supporting data for clinical trials. We expect to provide the preclinical data required to allow us to progress next-generation immune regulatory therapeutics to phase I trials.

Open access peer-reviewed publications and pre-prints. Here we will share our findings with the academic community.

Datasets. The data underpinning the publications will be shared openly and freely in relevant data repositories such as the Gene Expression Omnibus (GEO).
Animal models. We will continue to refine our experimental models and share these protocols within publications.

Who or what will benefit from these outputs, and how?

This project aims to both advance our basic science understanding of immune regulation as well as to provide the preclinical data required to translate cellular therapies and other immune regulatory therapies (biologics, small molecules) to clinical trials. The specific benefits are:

Benefit to patients: To develop and evaluate cellular immune regulatory therapies, biologics, and small molecules that promote immune tolerance. A specific focus will be on regulatory cellular therapies such as regulatory T cells with the aim of assessing next-generation cell products that are genetically modified to enhance their functionality. The data generated under this authority will provide the preclinical data required for MHRA approval for phase I/II trials. We have a proven track record in this regard with therapies developed under our PPLs progressing to three separate clinical trials.

Benefit to scientific community: We aim further characterise the physiological mechanisms that maintain immune tolerance. It is anticipated that this knowledge will contribute to the immunology with possible future opportunities for therapeutic development. Contribution to the field will be through publications and presentations, with the aim of 2-3 per year over the course of the lifetime of the PPL.

Benefit to 3Rs and patients: Our work on humanised mouse models for the evaluation of cellular therapies has formed the foundation for successful application to the MHRA in the UK and the FDA in the USA for progression of therapies to clinical trials. This therefore allows the acceleration of successful therapies to phase I trials without the need for assessment in non-human primates or other large animals.

How will you look to maximise the outputs of this work?

We will ensure maximum benefit by sharing all our findings, whether they are negative or positive, both in conference with publication of abstracts, and through peer-reviewed publications. Our methodologies for producing new experimental models, particularly humanised immune system mice, will be shared freely within our publications.

Our team has a strong record of collaboration across research groups, disciplines, and institutions and we believe that this is crucial to scientific advancement. We aim to continue this through our ongoing collaborations within academia within our institution, the UK, and internationally.

Our research has direct clinical relevance, and we will maintain our dissemination activities to the public and key stakeholders through press releases and patient and public engagement activities.

Species and numbers of animals expected to be used

- Mice: 30000
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The least sentient mammalian species that is appropriate for this work, namely mice and rats, will be used for this project. The availability of gene knockout and transgenic animals, that are absolutely central to the success of this project, means that the mouse is the preferred species to use for this work. Rats will be used for specific experiments related to vascularised transplants, where surgery is challenging in mice due to their size. Our mouse and rat experimental models are advanced enough that they have facilitated the direct translation of therapies from mouse to human, and have formed the basis of successful FDA and MHRA applications for advanced therapeutic approvals. For the majority of experiments, adult mice and rats will be used to allow for the transplants to be technically successful, however for a very small number of experiments where engraftment of mice with cells is more successful at a younger age, experiments will start with neonatal mice that are then transplanted with tissues as adults.

Typically, what will be done to an animal used in your project?

Well-characterised inbred and genetically modified mouse and rat strains will be used to allow the impact of specific genes on rejection, immunisation, infection and tumour

development to be determined. Both wild type and genetically modified animals will be bred and subsequently undergo a variety of transplantation procedures. All transplants will be performed in a dedicated operating suite by fully-trained personal licence holders. Anaesthetic, analgesia and post-operative protocols have been developed with the institutional veterinary service and represent current best-practice. Recovery from surgery is closely monitored and animals return to normal behavior (movement, feeding, drinking) within twelve hours. All other non-surgical procedures (such as immunisation) are performed in a dedicated procedure room under aseptic conditions, with animals monitored frequently post-procedure. Some procedures may be performed using human cells or tissue to emulate human immune responses. The surgical transplantation models used in this Project are the most advanced available and the result of decades of experience with their use by our group. Viral infection models are performed in dedicated areas in order to protect other mice. Occasionally, mice are irradiated in order to reconstitute their haematopoietic system with other genetic strains. Group sizes depend on the number of experimental groups and range between 4-10 mice depending on the type of experiment.

Typically, animals are prepared for donation of serum, cells, tissues or organs. This will involve intravenous or intraperitoneal injections. Animals receiving cell transplants will receive these through the intravenous or intraperitoneal route. In some cases these mice may also receive an injection of tumour cells or be irradiated before cell injection. Cell injection is sometimes performed to model graft- versus-host disease. Mice or rats receiving skin transplants will have a general anaesthetic for the procedure and have a patch of their skin replaced with donor skin from a different mouse or human.

Some mice may have their immune systems modified using a virus before skin transplantation, while others may be irradiated before transplantation to provide an environment for cell engraftment. Animals may be treated before or after skin or cell transplantation with agents that modify immune responses by topical, subcutaneous, intravenous, or intraperitoneal routes. Monitoring of transplant survival requires simple visual inspection (skin grafts) or tail bleeding to determine cellular phenotype and serum levels of therapeutic molecules, or an assessment of weight loss over time. To model the changes in blood flow that happen in transplantation, in some animals the kidney vessels may be clamped for a short period of time. Tumour monitoring is performed visually and by measurement of subcutaneous growth. At the end of the experiment (which is at the time of complete rejection or up to 100 days after transplantation) the animals will be humanely killed and transplants and other tissues recovered for histological, cellular,molecular and genetic analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts
The procedures in this project are safe and have been optimised over a number of years with veterinary input. Intravenous, intraperitoneal, and subcuticular injection is transiently painful but mice are normal shortly after the procedure. Skin transplantation is performed under general anaesthetic. Postoperative pain is possible but controlled with analgesia. Reduced mobility over the first 1-2 weeks after skin transplantation is possible as a result of the bandaging but this is temporary. Weight loss is also possible due to the bandaging, but this resolves soon after bandage removal. During the skin rejection process a local inflammatory response at the site of the transplant will occur, however animals do not generally become unwell with this.

Adverse effects
A number of very uncommon adverse effects may develop in animals as a result of surgery, immunisation, irradiation, infection, tumour implantation, or administration of drugs. However these adverse events will be recognised early before the animal is in significant distress and steps taken to prevent any suffering. Injection of virus may be associated with a short-term systemic illness characterised by slight listlessness for a period not exceeding 48 hours. The administration of immune cells may result in the development of autoimmunity or graft-versus-host disease that results in weight loss, hair loss, pallor, or hunching. Treatment of animals with drugs or antibodies to modulate immune responses is generally safe. Very rarely there is a risk of toxicity that may result in weight loss, hunching, and reduced mobility. There is a small risk of injury to the pharynx, oesophagus or larynx/trachea with oral gavage which may include inadvertent injection of substances into the lungs. However this is extremely rare (under 0.1% of animals). Very rarely (under 0.1% of animals), an air embolus may propagate after intravenous injection. Our experience of intraperitoneal and subcutaneous injections is that these techniques are not typically associated with any adverse effects. After skin transplantation and in typically less than 5% of cases, wound dehiscence may occur prior to healing. If this occurs, the recipient will be re-sutured if uninfected once under general anaesthesia. In our experience wound infection after surgery is very rare and has no cases have been observed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Subthreshold: 43%

Mild: 31%

Moderate: 26%

What will happen to animals at the end of this project?

- Killed

Replacement

**State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?**

The use of animals will allow us to study how the immune system reacts to foreign cells and tissues. The immune system is complex with many arms that act together as a system throughout many organs and thus its entire function cannot be recapitulated outside of an animal. For example, it is not possible to recreate the dynamic migration of cells between lymph nodes, the blood, and transplanted tissues in vitro. Studying immune responses in animals systems would facilitate an understanding of how the immune system behaves normally, how rejection takes place, and how it can be regulated therapeutically. Additionally, because the genetic makeup of rodents is very well characterised, it is possible to recreate very specific transplantation models in order to reduce confounding

factors. The availability of genetically modified animals allows the assessment of how changing only a single factor modifies the immune response.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we avoid the use of animals and prefer to analyse human tissues, for example using our new spatial genomic approaches which facilitate the analysis of archival human tissue biopsies. We have developed methodology to generate cellular therapies in vitro, and use in vitro techniques to explore specific elements of immune function such as cell migration and proliferation. We are also moving to expand our use of humanised mouse models to replace and reduce our reliance on pure mouse models. We are working on developing ‘organoid’ models which can recreate some elements of a whole organ system in the lab using cells from human donors.

In an effort to both improve the translatability of our research and replace animals, we have developed humanised mouse models. Here, immunodeficient mice and rats can be engrafted with human cells to allow the study of human immune responses without the need for larger animal models. Using these techniques, we have replaced the requirement for mice to provide donor organs with tissues obtained from discarded human tissue (obtained with full ethical approval and patient consent).

Why were they not suitable?

To date, it has not possible to recreate in vitro the complex micro and macroenvironments that are present within a transplanted organ in the context of a whole animal or replicate the communication that occurs in vivo between the transplant and the lymphoid system. Our in vitro systems allow the interrogation and assessment of only a small number of dimensions related to the immune response and cannot recapitulate both the innate and adaptive responses. Our in vitro migration assays can only assess migration of cells between two areas, and not the entire circuits present in vivo. Our 'organ on a chip' techniques provide the ability to assess some immune responses in the context of a section of human tissue but cannot yet provide the whole spectrum of immune responses over time with all the input from other lymphoid 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?

Animal numbers are estimated based on our knowledge of how many animals have been used in our previous experiments/PPL and the planned experiments to be performed on this PPL according to the projects that have received peer-reviewed funding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the majority of experiments, inbred strains of mice will be used and the strain combinations selected to allow a high chance of success. Experiments are designed to include clear objectives and rationale, appropriate controls, single variables in experimental groups, and observer blinding. The NC3Rs EDA is used where possible to help in experimental design. Control groups are often shared across multiple contemporaneous assays in order to reduce the number of mice required.

Where possible, we are moving towards in vivo assays where transplant rejection data may be qualitative rather than quantitative, therefore reducing group sizes. For example, the degree of rejection may be assessed in a single mouse based on in depth histological analysis of the tissue e.g. evaluation of the cellular infiltrate at a given time point rather than through obtaining transplant loss data. These qualitative methodologies are being optimised in a view to reducing our reliance on the use of large groups of mice in which transplant loss is the measurable end point. Similarly, we now use serial non- invasive in vivo imaging whenever possible in order to reduce the number of animals per experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Good colony management ensures that only those animals required for experiments are produced. A database is used to provide data for any breeding calculations.
We follow best practice in project organisation, management and data analysis helps us to keep the number of animals to a minimum. Where possible, we perform smaller pilot studies to confirm that our methodology is feasible before embarking on larger studies that may risk experimental failure.

We ensure good sharing of animals across our research group so that numerous organs can be harvested post mortem, resulting in fewer animals being needed by the group. For example, when skin is retrieved for transplantation, the blood or other organs removed as part of the retrieval process can be used to pre-treat another group of recipient mice and the spleens used for in vitro assays.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice and rats will be used for this project. These are the least sentient mammalian species that is appropriate for this work as the transplantation and experimental procedures require an animal of a certain size for technical success. While our previous work included heart, vessel and islet transplants, we have now refined our techniques to allow for cell or skin transplant models to provide as much information as possible through new in-depth analysis techniques including spatial transcriptomic profiling. Skin transplantation is the least painful of all the tissue transplantation techniques we have used in the past. We have also eliminated double transplantation with heart and skin, as we are now able to gain the required data from skin transplants alone.

Rats are up to ten times larger than mice, providing an advantage for technically demanding surgical studies. In particular, their size facilitates the transplantation of human grafts that require immediate vascularization, such as human skin. The use of these animals in transplantation studies is therefore a step closer towards clinical translation, allowing the evaluation of novel therapeutics in a unique vascularised human transplant context and therefore with the potential to obviate the need for non- human primate studies (such as those of kidney transplantation).

The ability to recreate a human immune system within immunodeficient mice and rats is a powerful steppingstone towards translation of therapies, evidenced by our ability to progress directly from mouse experiments to a clinical trial without experimentation on larger animal species (e.g. cynomolgus kidney transplantation).

Why can't you use animals that are less sentient?

A mammal is required to ensure a whole functional immune system is studied. Animals smaller than mice do not allow for the transplantation surgical procedures required to be performed. Immature or terminally anaesthetised animals do not allow for the important time course analyses, including transplant survival and graft-versus-host disease development.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We ensure that animals are handled with care to ensure minimal distress, using polycarbonate handling tubes. Our routes of administration are those which cause the least pain or distress but still achieve our scientific aims. We no longer utilise footpad injections for immunisation. When an extended period of injections are required, we will make use of implanted pumps to reduce the stress from repeated handling and injections. Irradiation, performed to deplete the immune system, is always delivered using split dosage to minimise distress and side effects. For our skin transplantation, we have developed flexible bandaging techniques that eliminate the need for immobilisation of the animal. Pain is monitored post-operatively using the NC3Rs Grimace Scale and adequate analgesia provided as and when required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance is collected from the NC3Rs (www.nc3rs.org.uk), LASA (www.lasa.co.uk), and Norecopa (norecopa.no). The establishment's Named Information Officer ensures that current guidance is disseminated to researchers. The NC3Rs has a webinar on 'Systematic reviews of animal studies' which provides an overview of the tools available such as the Systematic Review Facility (SyRF) which can help support meta-analysis of animal studies. The NC3Rs website provides many resources to help with further refinement such as advice and guidance on common procedures such as blood sampling and the evidence base to support the single use of needles (which we have always ensured is standard of care in our experiments). The NC3Rs also has information relating to non-aversive mouse handling, bleeding, genetically altered mouse breeding, and grimace scales which are helpful for all the experiments we perform.

By keeping up to date with the NC3Rs through their newsletter and website, I ensure that any new e- learning resources are disseminated to my research group. I also follow the

NC3Rs/LASA training material on the Procedures with Care which covers the administration of substances to mice and rats and aseptic technique in surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of the group are encouraged to attend seminars hosted by the establishment where new and refined developments are disseminated. Researchers are also required to stay up to date with the literature in order to learn of new scientific techniques that may replace animal work, and also keep an eye on the NC3Rs website for any new findings or advice as well as events, also subscribing to their newsletter. Regular discussion with animal care staff as well as the NC3Rs Regional Programme Manager complement the other sources of information.

46. Mechanisms of Acute and Chronic Inflammation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Inflammation, Lungs, Infection, Asthma, Fibrosis

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall objective of our investigations is to identify molecules and mechanisms that start, progress and end the inflammatory response in acute and chronic lung diseases/respiratory conditions (such as asthma, chronic obstructive pulmonary disease, lung fibrosis and respiratory tract 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.

Why is it important to undertake this work?

The inflammatory response involves directing specific white blood cells to sites of disease and damage, where they interact with other cells and release substances which allow the surrounding cells to divide and fight the disease and/or repair the damaged tissue. However, how the cells are directed to and function at sites of disease and damage are not well understood.

Inflammation is a normal biological process that the body uses to protect itself against diseases and for restoring damaged tissues back to normal functions. However, when inflammatory processes are poorly controlled or directed against normal bodily functions,

they are harmful to the affected individual and result in diseases such as Asthma, Lung Fibrosis and Chronic obstructive pulmonary disease.

Understanding the mechanisms involved in the inflammatory response is fundamental to analysing the processes of infectious disease control on the one hand and inflammatory diseases on the other. This understanding is critical for the design of new drugs for common inflammatory diseases for which there are currently few effective treatments

What outputs do you think you will see at the end of this project?

This work is expected to provide new information about mechanism of inflammation in lung diseases and how these can be manipulated for benefit.

The expected primary benefit is the publication of high-impact scientific papers about inflammatory mechanisms in lung diseases and information that will impact clinical management of such diseases.

Who or what will benefit from these outputs, and how?

Throughout the life of this project, data produced will be presented at national and international conferences and published in academic journals. The new information will improve our understanding of mechanisms of inflammation in airway diseases and how they can be manipulated to promote lung repair rather than further damage and thus restore normal lung function. We will also promote and publish any refinements or best practice we identify during this project. We will make post-mortem tissue available to collaborators.

In the medium term, the clinic will benefit from the identification of markers to allow better identification of patient subgroups, while the pharmaceutical industry will be interested in potential novel therapeutic targets we identify.

The long-term benefits of this study are that data generated will have far-reaching implications for the treatment of respiratory diseases, benefitting patients and clinicians by contributing to the development of novel treatments for acute and chronic inflammatory diseases, and new preventative strategies for childhood disease such as asthma, which will ultimately reduce the economic and health burden caused by such diseases.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings.

Additionally, we will take as many tissues as possible at post-mortem and will make them available to other researchers immediately or in future by biobanking them.

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

It is critical to perform these studies in mammals since there are significant differences between the biological systems of frogs and fish to that of humans. Mice are the least sentient animal that display many of the important features seen in human diseases. Furthermore, for genes which instruct the cell how to make proteins, mice and human are on average 85% similar. Therefore, we will also use genetically altered mice, as well established and sophisticated techniques exist that allows us to switch genes on and off and in specific cells. This enables us to examine the role of genetic defects too. Additionally, conditions like asthma and respiratory infections which occur in both children and adults present differently in these age groups and have different underlying mechanisms, which are also observed in mice. Therefore, we will use neonatal (new-born) and adult mice in our studies as it is important that we use age appropriate mice to address our research questions.

Typically, what will be done to an animal used in your project?

Most animals will be exposed via the nose to either an allergen (3 or 5 times weekly for up to 5 weeks) or a pathogen (on one or two occasions) or an agent that causes lung fibrosis (on one occasion), along with an additional substance which will alter the development of the disease. Disease progression and animal welfare will be monitored at least once daily and will include weighing, and assessment of body condition and behavior, until satisfied that there are no welfare concerns, then checks will be reduced to not less than twice weekly. At critical time points, under general anesthesia without recovery, the animals' lungs and/or blood vessels will be imaged, or lung function measured, before cells and tissues are collected. For animals exposed to an allergen the experimental duration will either be 3 or 5 weeks; for those exposed to a pathogen 10 days; and those exposed to an agent that causes lung fibrosis 7, 21 or 42 days.

Prior to the above, some animals will undergo removal of bone marrow cells and subsequent replacement of such cells (bone marrow ablation and reconstitution), or surgical removal of the spleen, to modify their immune systems.

What are the expected impacts and/or adverse effects for the animals during your project?

The following procedures are expected to cause some moderated discomfort, that animals will fully recover from quickly. They will be given painkillers and post-procedural treatments and checks just like people are; bone marrow ablation and reconstitution (tiredness and reduced appetite), removal of the spleen (pain) and pathogen exposure or lung fibrosis (weight loss). Other procedures, such as administration of allergen or substances, are expected to cause minimal but transient discomfort with no lasting harm. During procedures under general anaesthesia without recovery, the depth of anaesthesia will be sufficiently deep that the animal will feel no pain. We do not anticipate any severe adverse events.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

5% Non-recovery

45% mild

50% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals in our project because lung diseases and inflammation are complex processes that involve many different tissues and cells working together, which cannot be adequately or fully modelled by computational or in vitro (non-animal) studies alone. We do not fully understand these multifaceted processes or how the complex interactions between different parts of the body, cells, tissues and the environment cooperate, so unless we study animals as a whole, we cannot learn how to prevent and treat these conditions.

Which non-animal alternatives did you consider for use in this project?

In our work we use many different types of techniques and experiments to answer our scientific questions. Throughout the project, where possible, we will complement the in vivo (live animal) work with ex vivo (non-live animal) techniques such as precision cut lung slices, and mouse or human derived cell and tissue culture systems. When suitable non-animal experimentation using in vitro and in silico (computer) techniques will be used including;

Cell culture - submerged, air-liquid interface cultures and co-cultures of primary isolated human cells and cell lines.

lung-on-a chip - using primary isolated human cells and cell lines human samples databases and data sets

However, these are not adequate to fully replace all animal experimentation.

Why were they not suitable?
Such methods do not consider complex interactions that occur between integrated biological systems (for example respiratory, vascular and immune systems) and the external environment in the way that a fully intact organism does. Different cell types require different growth conditions some of which are incompatible with each, rendering full modelling impossible. Technologies to model pressure, and air and blood flow are still in development are not currently robust enough to meet needs. We will continue to monitor the field for novel techniques using non-animal materials and will implement them, where appropriate, as they arise.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 projected number of animals reflect the number necessary to achieve the scientific objectives outlined in the programme of work described in this application. We have estimated mice based on the anticipated numbers of experiments, the numbers of experimental groups and the numbers of mice in each group. The estimates are based on similar studies we have conducted in the past using similar protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3Rs Experimental Design Assistant and consult with the college Statistical Service to design experiments with the fewest animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Genetically modified mice will only be maintained whilst there is a justified use for their continued breeding. We will breed in ways that minimise waste and ensure the majority of animals can be used in experiments.

We are now using the Precision Cut Lung Slice technique, an ex-vivo technique which allows imaging of live viable lungs (up to 7 days from removal). This results in a reduction as multiple slices can be obtained from a single mouse.

We have refined our assays such that we can measure multiple parameters in each animal, thus maximising the information gained from each experimental group. Combining tests in the same mice allows the interpretation of data to be correlated directly, rather than inferred. This allows us to carry out more advanced statistical analysis to detect correlated trends between assays. Directly correlated effects have a greater sensitivity as the major source of variance (between mice) is removed. 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.
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen the smallest animal possible to represent human disease. While the model chosen closely represents the important features of the human disease in the treated animals, they are the least severe and do not promote undue distress to the mice. Where possible, we administer substances locally rather than systemically (through the entire body) to restrict systemic side effects. We constantly monitor animals for signs of ill health and work closely with animal care staff and veterinary surgeons to ensure the best possible husbandry and welfare for mice under procedure. Anaesthesia and analgesia (pain relief) are used routinely in all animals when required and appropriate. Suffering is minimised by procedures being carried out by highly skilled and experienced scientists.

Why can’t you use animals that are less sentient?

It is critical to perform these studies in mammals since there are significant differences between the physiological systems of frogs and fish to that of humans. The mouse is the lowest mammalian species in which the full range of genetic and physiological manipulations necessary for the investigation of lung disease and inflammation can be achieved.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

While the model chosen closely represents the features of the human disease in the treated animals, they are the least severe and do not promote undue distress to the mice. We will frequently monitor animals for signs of ill health and work closely with the NACWO and veterinary surgeon to ensure the best possible husbandry and welfare for mice under procedure. Refinement measures carried out in this project will include: use of anaesthesia and analgesia whenever possible as well as enrichment of the animal's environment. We will increase monitoring if any adverse effect occurs and consult the NACWO and veterinary surgeon and offer pain relief, treatment, stop the experiment or humanely kill animals as appropriate. Through the project we will continue to look for new ways to refine our experimental protocols and make animals more comfortable. This could include when appropriate milder models (effects or earlier end-points) along with milder administration routes, lower administration volumes and/or reduces administration frequencies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Frequent communications (newsletters and meetings) are produced within the institution on the advances in the 3Rs and their implementation. All staff working under this licence will be signed up to receive these. Advice regarding the well-being of animals will be sought from the vets and animal care staff regularly.

We will follow external sources including National Centre for Replacement Refinement and Reduction of Animals in Research resources (website https://www.nc3rs.org.uk/).

47. Understanding Common Human Neurodegenerative Diseases

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

mechanisms of neurodegeneration, propagation of protein aggregates, Alzheimer's disease, Parkinson's disease

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to understand more about the mechanisms of neurodegenerative diseases, in order to finding new targets which could lead to the development of mechanism-based therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases are among the most common age-related human diseases. Alzheimer's disease is characterized by dementia, whereas Parkinson's disease is primarily a movement disorder. Existing treatments only treat disease symptoms without modifying the underlying disease process.

What outputs do you think you will see at the end of this project?

The assembly of a small number of proteins into insoluble filaments is the gain-of-toxic function mechanism that underlies age-related neurodegenerative diseases. These filaments are not just clumps of unfolded proteins. Our on-going electron cryo-microscopy studies have shown that individual molecules form specific contacts to give rise to long helical filaments. Tau filaments are associated with several diseases, including Alzheimer's disease, and the types of contacts they make are characteristic of particular types of disease. Another protein, alpha-synuclein, also forms inclusions in brain cells and is associated with diseases, such as Parkinson's disease and multiple system atrophy. It also forms filament structures that are specific for each pathology.

We are now developing methods by which to assemble recombinant tau and alpha-synuclein into filaments with structures like those of filaments from human brain. Depending on the conditions, we have shown that recombinant tau (297-391) can assemble into filaments identical to those of the paired helical filaments of Alzheimer's disease or type II filaments of chronic traumatic encephalopathy. We are developing methods for reproducing other folds.

This work opens the way to producing transgenic mouse models with filament structures like those from human brain. None of the existing mouse models have developed such filaments. This may explain why mouse lines have been imperfect models of human disease. The new possibilities provide a substantial refinement. We are launching an ambitious and extensive programme of research along these lines. At present, we are in the lead worldwide as far as high-resolution structure determination of disease filaments goes. We will extend this lead to the generation and characterisation of transgenic mouse models of a number of human neurodegenerative diseases.

Therefore our outputs will be new improved mouse models of human disease which have filament structures like those found in human brain. All new information will be disseminated in peer-reviewed open access publications and at presentations at scientific meetings.

Who or what will benefit from these outputs, and how?

All new information generated during our project will be made available to the scientific community and will benefit all researchers with an interest in human degenerative diseases especially tauopathies and synucleinopathies and in protein aggregation in general.

Once published, we will make the new mouse lines available to academic and pharmaceutical laboratories. These new improved mouse models will be used for the testing of novel biomarkers and mechanism-based treatments of neurodegenerative diseases. In the short term it will impact research and development in the field and in the long term this knowledge will guide the development of novel biomarkers and mechanism-based therapies.

How will you look to maximise the outputs of this work?

By publishing in high-impact journals, by talking about this work in scientific meetings and by collaborating with others. In addition, we will make these lines freely available, without requiring authorship.

Species and numbers of animals expected to be used

- Mice: 45,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Human neurodegenerative diseases are diseases of the central nervous system. Mice have a central nervous structure that is comparable to that of humans. They are open to genetic manipulation and their generation time is reasonable.

The majority of mice used will be adults mice and aged mice to study disease progression. We will also maintain breeding colonies and making new transgenic lines and cryopreserving existing transgenic lines which means that all life stages will be used in this project.

Typically, what will be done to an animal used in your project?

Mice will be housed in a social environment whenever possible and provided with environmental enrichment such as chew sticks, fun tubes, nesting material and platforms.

Some mice will aged to allow development of the neurodegeneration. These mice may experience weight loss and hind limb impairment as the disease progresses.

Some mice will undergo behavioural tests to check for neurodegeneration such as motor function or memory tests. These tests are non-invasive and there are no expected adverse effects.

We may administer substances by a variety of routes, choosing the most suitable and least invasive route possible. Some of the routes will be non-invasive such as food or water. Other routes such as injection (under the skin, into the peritoneal cavity, into a vein, into a muscle) or gavage will cause mice transient pain and/or transient stress.

Some mice may receive surgical injections into defined areas of the brain. Surgery mice will be given pain relief prior to recovery from anaesthesia and whenever necessary to alleviate pain as advised by the veterinarian. Following the administration of substances mice will be aged to observe the possible development of neurodegeneration in brain and spinal cord, in which case these mice will experience weight loss and pain due to progressive hind limb impairment.

At the end of experiments, mice will be humanely killed and samples collected for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Some mice will develop symptoms of neurodegenerative disease, which may manifest themselves by weight loss, muscle weakness and mobility problems. Ageing mice may experience pain due to age- related diseases such as neoplastic and chronic degenerative non-neoplastic diseases.

Mice will experience transient pain post injection. They will experience stress and post-operative pain due to surgery. Post surgical pain is limited up to 24/48 hours and pain relief is given to the mice.

We have monitoring programmes in place for all animals to assess health status in order to avoid animals experiencing unnecessary adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

60% of mice will not experience any pain and are classed as sub-threshold severity.

10% of the animals will experience some pain which will be mild severity such as an injection.

30% of animals will experience pain such as progressive back leg disability and are classed as moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 identification and evaluation of genetic and pharmacological modifiers of neurodegeneration requires the use of experimental animal models.

The brain structure of all mammals is similar and so the mouse is best suited for this work since, of all existing models of neurodegeneration, mouse models are the closest to the human diseases.

Which non-animal alternatives did you consider for use in this project?

We are studying the structures of tau and alpha-synuclein filaments made in the test tube by electron microscopy to obtain high resolution images. We have established cell assays to study mechanisms of uptake and cell-to-cell propagation of tau and alpha-synuclein assemblies of defined structure in vitro. These data are taken into account when designing animal experiments.

Why were they not suitable?

We do carry out experiments in cell lines in our laboratory, but whilst this will provide some information, cultured cells do not provide physiological conditions or the complex

interactions amongst different cell types. Tissues can also be used but only provide an isolated response, which is not completely representative of in vivo responses, because inducers and modifiers of pathology often involve metabolism and interplay among different 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?

Based on past experience we estimate that we will need around 8,000 mice by year. We maintain around 25 transgenic colonies. Our transgenic lines express one of three proteins (beta-amyloid, tau and alpha-synuclein) in a wild-type, mutant or truncated form. In other lines, the function of a mouse gene may be disrupted by gene knockout or modified by gene knockin. Some of these lines will be crossed with transgenic lines expressing putative modifiers of disease such as genes involved in inflammation or knockout or knockin transgenic lines.

Some mice will be used for breeding to maintain the colony. Some mice will be bred for tissue collection for ex vivo work. Some mice (in groups of 8 to 12) will be aged to allow neuropathology to develop prior to tissue analysis of a time course which will usually have four to eight time points. Some mice will be given pharmacological modifiers (groups of 8 to 12) and tissues collected for analysis.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All animal work is preceded by detailed studies in test tubes and cells. We will use the minimum number of animals needed to give a statistically significant result. A statistical expert will be consulted regarding experimental design and the most appropriate statistical analysis. Prior experiments and expertise with the animal model used will be taken into account when deciding on the number of animals needed for an experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mouse breeding will be carefully monitored to ensure that surplus animals are not generated. Wherever we use a new substance in mice we will conduct a small, time-limited pilot study.

Cryopreservation is used routinely to preserve important mouse lines and to remove the need to breed mice only to maintain a given line.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models of common neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, which we use express the human proteins known to be involved in the human diseases (beta-amyloid, tau and alpha-synuclein) and exhibit the essential features of the human diseases and as such they are the best models available for our studies.

We have a monitoring programme in place for all our animals to assess health status in order to avoid animals experiencing unnecessary adverse effects. There is an enhanced monitoring programme for older animals and animals which may develop weight loss and/or hind limb impairment to minimise suffering.

When new lines are generated and bred for the first time, animal technicians will be specifically informed and the first litters carefully monitored. Any untoward phenotype will be discussed with the NACWO, veterinarian and if appropriate, the Home Office inspector.

The surgery will be carried out under asepsic conditions and we will aim to follow "Guiding principles for preparing for and undertaking aseptic surgery" (2010) as closely as possible. Mice will be given analgesia prior to recovery from anaesthesia and whenever necessary to alleviate pain as advised by the veterinarian.

All animal experimentation conducted under this project licence will comply with the document entitled "Animal Usage Guidelines". This document has been adopted by the local Ethical Review Process in order to inform researchers of the bounds within which their animal work should be conducted, and to provide practical recommendations on various aspects of animal experimentation.

Why can't you use animals that are less sentient?

Mice are the least sentient representative species we can use: we need to use a mammalian species because it has a central nervous system comparable to ours and mice are a suitable model because we have the ability to manipulate mice using genetic means. The greatest risk factor for neurodegenerative diseases is ageing so mouse models will be aged to investigate the contribution of the ageing process in neurodegenerative disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Some of our mice develop motor impairment as they age and these mice will be closely monitored for difficulty satisfying hunger and thirst and for weight loss. Where appropriate food and drink will be provided on the cage floor.

In view of the progressive decline associated with ageing there will be increased vigilance both in monitoring indices of general health (weight, grooming, reactivity) and additional observations designed to detect specific problems such as the development of tumours.

For administration of a substance the route of administration will depend on the solubility and bioavailability of the compound and the least invasive route that is suitable will be chosen.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use the NC3R website to ensure that we follow best practice. We use The Experimental Design Assistant (eda.nc3rs.org.uk) on the NC3R website to to check the design of our animal experiments and ensure that we have robust and reproducible data.

We follow the PREPARE (norecopa.no/prepare) planning guidelines and the checklist of the ARRIVE (arriveguidelines.org) guidelines to maximise the quality and reliability of our publications.

We also follow best practice for our surgery experiments as laid out in the LASA guidelines (lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep up to date with the current literature and subscribe to the NC3Rs newsletter to get the latest updates. We attend seminars and conferences where the 3Rs are discussed. We encourage the sharing of best practice between animal technicians.

All animal welfare issues are addressed and solutions implemented following discussions between the animal technicians, the NACWOs, the veterinarian and PIL holders.

48. Elucidating sources of contrast in quantitative brain MRI

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
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Magnetic Resonance Imaging, brain, histology, multiple sclerosis

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate how different components of the brain tissue contribute to the observed contrast in magnetic resonance images.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Magnetic Resonance Imaging (MRI) is a type of scan that uses strong magnetic fields and radio waves to produce detailed images of the inside of the body. It is for example routinely applied to image the brain of healthy people and of patients. The characterization of one particular type of tissue in the brain, i.e. the white matter, is important for diagnosing disease (such as for example Multiple Sclerosis). While MRI is very good in depicting

lesions in the brain ("qualitative assessment"), clinicians are now interested in quantitative information such as length or volume of brain cells to provide a more refined diagnosis. The structure of white matter, however, is complex, which makes it challenging to interpret the MRI images in an accurate and quantitative manner. We therefore need to study the relationship between imaging findings and anatomical features in an animal model such as the mouse, where we can easily compare our MRI findings with histology. This would not be possible in humans. Histology can then be related to the MR images in a novel way by spanning a range from nanometer to millimeter.

What outputs do you think you will see at the end of this project?

To date, quantitative readouts ('imaging biomarkers') from MRI are indirect, meaning we are measuring physical properties (such as relaxation times or diffusion parameters etc.) from which we infer physiological or anatomical properties. The estimated parameters will be affected by tissue properties AND potentially by the experimental configuration. It is therefore important to understand the influence and contribution of both parts. This process becomes a fundamental aspect for developing these imaging biomarkers and it will be ultimately essential for their application on patients in the clinics. The output of this project will therefore lay the foundation for answering major open questions in multi-scale imaging (i.e. covering resolution from µm- to mm-range) and in MRI.

Who or what will benefit from these outputs, and how?

The proposed experimental design (i.e. manipulation with the cuprizone model) together with the cutting edge MRI and electron-microscopy technologies used in the present study will generate an extremely valuable multimodal dataset. We expect the outputs of our study to be beneficial on both short and long terms. Initially, throughout the duration of the research project, methods, analyses and findings will be presented internally and externally trough conference reports for the benefit of the scientific community to support collaboration and design of new research studies. Furthermore, we will use a combination of approaches providing complementary information to gain a deeper understanding of the biophysical origins of differences in the estimated MRI parameters thus enhancing the interpretation of quantitative MRI. Hence, on the long term, we hope outputs from the present study will help devising biophysical models capable of achieving a better biological accuracy and perhaps, enhanced diagnostic power boosting MRI clinical utility.

How will you look to maximise the outputs of this work?

Throughout the duration of the research project, study results will be reported using peer reviewed journals and conference reports. Where possible original, peer-reviewed research publications will be made freely available and open access to research data will be supported. Furthermore, collaboration among research groups will be promoted. Data analysis will be reported and made available to enhance reproducibility and replicability. Finally, the PhD thesis will be made freely available from the EThOS repository

Species and numbers of animals expected to be used

- Mice: 70

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
The project will use adult wild type C57Bl/6 mice as they are one of the predominantly used species in biomedical research. Furthermore, C57Bl/6 is the strain where cuprizone induced demyelination has been consistently reported. It is therefore the most utilized strain in studies using the cuprizone diet.

Typically, what will be done to an animal used in your project?

The project will use two groups of mice, only one of which will be fed with a Cuprizone supplemented diet for a duration of six weeks. Both groups however will be subjected to two MRI scans, six weeks apart, typically lasting 2-3h. In rare circumstances, due to technical difficulties with equipment, a third MRI scan may be performed. In either case, the second or third scanning session will be performed under terminal anaesthesia. A clinically approved MRI contrast agent might be given during the imaging session.

What are the expected impacts and/or adverse effects for the animals during your project?

Cuprizone feeding has been associated with weight loss. This is expected to happen especially in the first two weeks of diet followed then by a gradual increase in body weight, which however remains below the baseline. Behavioural phenotype associated to cuprizone feeding include motor limitations such as tremors, abnormal walking and reduced coordination, spatial memory, reduction in social interaction with peers, environment exploration and spontaneous motility. Administration of cuprizone supplemented diet will be limited to a short period of time therefore preventing permanent damage.

MR imaging is performed under general anaesthesia. While the adverse effects of general anaesthesia are rare, the mice will typically undergo two MRI sessions six weeks apart under general anaesthesia. The animals will not be recovered after the second imaging session. If for technical reasons a third scan is required, it will be conducted under terminal anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for all animals will be *moderate*.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are essential to understand the observed imaging contrast under in vivo conditions as mimicks the clinical scenario. Furthermore, the ability to compare the in vivo measurements with histological “ground truth” analyses in a controlled setting is a fundamental aspect and would not be possible or ethical to perform in humans.

Which non-animal alternatives did you consider for use in this project?

We will always make use of non-sentient phantoms and/or cadavers and ex vivo tissue in the first phase of the development.

Why were they not suitable?

None of these alternative settings reflect the complexity of the in vivo situation (as also found in patients), where there is interplay between motion (i.e. cardiac and respiratory motion, blood flow) and complex (magnetic) properties associated to living brain 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?

Authority is being sought for a defined study, for which the number of required animals was determined using power calculations (n=20). An additional group (n=20) has been added for validation after the developmental phase on non-sentient phantoms / under ex vivo conditions.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Minimizing the number of animals will be achieved by moving to in vivo testing / validation only once all imaging sequence are fully tested on non-sentient phantoms / under ex vivo conditions. We will adhere to a strict workflow to achieve our scientific goals while minimizing animal usage. The NC3R's Experimental Design Assistant has been used to design the study while minimizing the total number of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As mentioned before, we will always make use of non-sentient phantoms and/or cadavers and ex vivo tissue to ensure that all imaging techniques are working before the in vivo application.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 because this species provides sufficient similarity in health and disease to man. The proposed in vivo imaging is non-invasive in nature but requires for the animal to be anaesthetized. The dietary intervention is required to generate the disease model but will be limited to a short period of time therefore preventing permanent damage.

Why can’t you use animals that are less sentient?

Mice are the mainstay of biomedical research. In order to elucidate the imaging contrast observed in MRI and to perform histological validation, these models are essential.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As mentioned, the proposed in vivo imaging is non-invasive in nature, but requires for the animal to be anaesthetized. All animals will receive additional heat and fluid support during and after the imaging examination to aid recovery from anaesthesia and will be closely monitored until fully recovered. During imaging sessions, ECG, respiration and body temperature are continuously monitored. All cages include environmental enrichment and animals are kept in social groups whenever this is compatible with our objective. Cuprizone feeding has been associated with weight loss. This is expected to happen especially in the first two weeks of diet followed then by a gradual increase of body weight, which however remains below the baseline. We will therefore weigh the animals at least three times a week and document it.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website has a repository on topic-specific resources, including anaesthesia and analgesia, which are the most relevant topics. In addition, regular updates on training and best practice guidance is disseminated via the mailing lists mentioned above.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have university-wide mailing lists informing on advances in the 3Rs and on new training opportunities in this area. Furthermore, we are in close and regular contact with the University Veterinary Officer, with whom we regularly review our procedures. This represents an effective mechanism to convey and translate any new advances into code of practice.

49. Delivery, efficacy, and safety of nucleic acid therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

Key words

small interfering RNA (siRNA), antisense oligonucleotide (ASO), gene therapy, nucleic acid chemistry, nucleic acid therapy

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Determine the effectiveness of delivery, tissue / cell distribution, efficacy and safety of nucleic acid therapeutics with different chemical modifications, formulations and routes of administration

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Nucleic acid (RNA and DNA) therapeutics are a promising, relatively new class of medications offering the advantages of highly precise effects, rapid development time,

broad target range, and durable action. Several nucleic acid therapeutics have received regulatory approval and offer unparalleled therapeutic effects. However, enabling the application of nucleic acid therapeutics to a broader class of diseases requires understanding of how nucleic acid chemistry, formulation, and route of administration affect delivery and efficacy. Thus, this work seeks to characterise the effects of nucleic acid characteristics on these outcomes.

What outputs do you think you will see at the end of this project?

- Delivery of nucleic acid therapeutics to a range of tissues, such as CNS, kidney and heart
- Identification of novel nucleic acid chemical modifications or delivery methods that enhance the tissue/cell type specificity of delivery
- Identification of novel nucleic acid chemical modifications or delivery methods that augment the efficacy of genetic targeting
- Establish the feasibility of modulating specific disease-relevant target gene

Who or what will benefit from these outputs, and how?

Ultimately, human patients with diseases which could be treated by nucleic acid therapies will benefit by our demonstration of safe, effective therapeutic delivery. Our projects are prioritised to maximise the real clinical benefit. In the intermediate term, collaboration partners (academic institutions and biotech companies) will benefit from our research on how to improve the delivery, precision, effectiveness and safety of therapeutics. Concretely, we expect to identify specific nucleic acid chemical modifications, conjugated molecules, formulations, and delivery routes that improve the specific delivery (target organ/cell type compared to off-target), require less nucleic acid therapeutic compound to be delivered, or compound to be delivered less frequently (due to improved stability, or more specific distribution, or more potent genetic effects), and/or have an improved safety profile (e.g. reduced immune response), compared to existing therapeutic options. Furthermore, we expect to establish the feasibility of modulating specific disease-relevant target genes for future therapeutic development and application.

How will you look to maximise the outputs of this work?

- Our central mission is to accelerate the development and implementation of nucleic acid therapeutics. We will achieve this goal by:
- Disseminating our results to collaboration partners (academic, clinical, industrial, and charity) and the scientific community
- Organising conferences to exchange ideas and disseminate findings
- Utilising social media and other public relations channels to publicise our research and results Publication of results in peer-reviewed scientific journals

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The primary endpoints of the work are:

- to determine the distribution of nucleic acid-based compounds in different organs and cell types to determine the effectiveness of gene modulation in target organs and cell types
- to determine whether the compounds cause adverse effects

Each of these endpoints requires intact physiology, including functioning circulation, immune system, and organ systems. The distribution of compounds depends largely on route of delivery, physiological processes (e.g. liver metabolism and kidney excretion), chemical modifications enhancing stability and cell uptake, minimising protein interactions and immune response), and formulation (e.g. linking to other molecules to improve distribution). We will investigate different chemical modifications and formulations to promote delivery to target organs and cell types.Our aim in this Project is not to carry out dose-escalation studies specifically designed to ascertain toxicity of a compound in vivo.

The ultimate purpose of the work is to develop therapies for use in humans. With that in mind, mice offer several advantages over other species:

- Physiology and organ systems are closer to humans than, for example, fish or flies
- Mice are well established for routes of delivery applicable to humans, such as intravenous, subcutaneous and intrathecal injections

We will primarily use adult mice (age 2-6 months), to avoid confounding effects of development on physiology. In cases where the target gene is relevant in the context of early onset disease, we may use juvenile mice (3-8 weeks) for delivery outside of the nervous system.

Typically, what will be done to an animal used in your project?

Typically, an animal will receive a single dose of a double-stranded nucleic acid (e.g. siRNA), or four doses of a single stranded nucleic acid (e.g. ASO) 1 week apart. Compounds will only be delivered after extensive purification, chemical quality control, and testing in lab-grown cells to ensure effectiveness, precision, and minimal toxicity. The route of administration could be subcutaneous (s.c., under the skin), intravenous (i.v., into the vein), intraperitoneal (i.p., into the abdomen), intramuscular (i.m., into the muscle), intrathecal (into the spinal fluid), or intracerebroventricular (i.c.v., into the brain fluid), but each animal will only receive a single compound through one route. For intrathecal and i.c.v. administration, animals will be sedated or anesthetised to enable accurate administration. Immediately following administration, animals will typically be housed in metabolic cages. Blood and urine will be sampled to establish the pharmacokinetic profile of the compound. Subsequently, blood will be sampled weekly to monitor immune response and liver and kidney toxicity. Dependant on observed adverse effects and/or selected gene(s) to be targeted, following sufficient incubation time (for example, 2 weeks to 6 months), animals will be euthanised for examination of their tissues for gene expression and pathology, including quantifying biodistribution of the compound.

What are the expected impacts and/or adverse effects for the animals during your project?

Nucleic acid compounds with established chemical structures, formulations, and delivery routes are likely to cause few adverse effects. The administration itself will likely cause mild transient distress due to handling and injection, as well as sedation/anaesthesia where required. Novel chemical structures, formulations, and administration routes may cause unexpected adverse effects, but the likelihood is low because of the well-understood mechanisms of action. The most likely adverse effects are liver or kidney toxicity (the sites of compound metabolism and excretion respectively) or inflammation. Adverse effects may also occur as a result of modulation of the target gene, but this is also unlikely since genetic targets are chosen based on the expected beneficial effects of their modulation. Adverse effects could occur via off-target control of unexpected genes; yet this is also unlikely as extensive investigation of this phenomenon will be carried out (e.g. using transcriptomics) in cell lines. Blood sampling will cause transient discomfort, but no lasting harm is expected.

Repeated blood sampling may in rare cases (<1%) cause soreness around the site of collection on the tail. Intrathecal and intracerebroventricular administration may cause death from over dose of anaesthesia (<1%), death because of a failure to recover from anaesthesia (<1%), procedure- associated infections (this is anticipated to be extremely rare, <1%). For mice receiving intrathecal/i.c.v. injections, there is a possibility of wound infection (<1%) or wound reopening (anticipated to occur in <5% of animals). Accidental damage to the nervous tissues during substance administration may lead to temporary or permanent nervous signs post-procedure (<2%). In some cases an adverse response to the substance may occur. This may include inflammation, altered behaviour, or seizures. Adverse reactions to substances are anticipated to occur in less than 5% of animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate (all animals)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The work aims to determine the distribution of compounds among organs and cell types, their efficacy, and their potential adverse effects. Each of these requires an intact mammalian physiology including circulation, immune system and organ system that are analogous to humans.

Which non-animal alternatives did you consider for use in this project?

All nucleic acid-based compounds will first be extensively tested in lab-grown cells to ensure their genetic precision and effectiveness and to predict their safety as much as possible based on markers of cellular stress and toxicity. Specifically, compounds will be introduced into one or more human cell lines at a range of doses. We will measure expression of the target gene (compared to non-targeting control) and also ultimately the entire transcriptome to evaluate specificity. We will also evaluate markers of cellular toxicity and death. We also considered lab-grown tissues derived from human stem cells.

These can replicate aspects of organ formation and have been investigated in the context of different organs including forebrain, kidney, and liver. They contain authentic cell types and replicate some aspects of physiology, such as kidney filtration, but not others, such as immune responses.

Why were they not suitable?

Lab-grown cells and tissues will be used to test compounds with respect to genetic efficacy and specificity, but cannot be used to assess compound distribution in the context of intact circulation, immune system, and inter-organ communication. For example, compounds cannot penetrate the interior of lab-grown tissues (e.g. organoids) due to a lack of circulation. Lab-grown tissues also have no utility in predicting physiological adverse effects (e.g. inflammatory response).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will, using extensive targeting and toxicology testing in immortalised and primary cells lines, prioritize compounds for evaluation in mice. In similar studies, statistically significant effects can be observed with 6-8 mice. We plan to administer up to 15 compounds per year (9 given systemically, 6 delivered to the CNS) at up to typically three dose levels, plus up to two negative controls (for example, receiving a non-targeting sequence with and/or without specific chemical modifications) on each occasion. More than one compound may be used to investigate the same genetic target. Both sexes will be used.

Thus, up to 5 treatment groups per compound x 8 replicates = 40 x 15 compounds = 600 mice x 2 sexes
= 1200 mice x 5 years = 6000 mice total

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the LASA/NC3Rs “Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals”. For initial screening studies, three compounds is the minimum to provide sufficient diversity and buffer and reduce the risk of failure. For dose-finding studies, three doses (low, medium, and high, based on established dose guidelines for the class of compound and route of administration, as well as effective dose in lab-grown cells) is the minimum number to determine the therapeutic window in which a

beneficial effect is observed with minimal adverse effects. Based on similar studies in the literature, 6-8 mice per group is the minimum required to obtain interpretable results, given the typical effect sizes and variances observed in previous studies of this type. We will use both sexes. Experimental design will be reconsidered for every compound/route and the required sample number calculated based on appropriate statistical tools and using current data on variability and effect size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- We will minimise variability, and therefore number of animals required to obtain interpretable results, by using inbred strains and ensuring good animal health and minimum stress. We will use animals of the same age and strain for each study. We will use small-scale initial pilot studies to determine the viability and best dose of lead compounds for each genetic target.

- We will avoid having to repeat experiments for additional data collection by collecting a wide range of tissues at the time of euthanasia

- We will avoid repeating experiments in cases where the effects of compounds with similar chemical structures have already been well characterised by us or others

- We will strive to disseminate negative results from our studies (i.e. cases where compounds were not effective or not well tolerated), to inform other groups and prevent them needlessly repeating studies with ineffective compounds

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild-type (non-genetically modified) mice, primarily of the C57BL/6J strain to ascertain compound efficacy. Typically, mice will receive one dose of a double-stranded nucleic acid (e.g. siRNA) or up to 4 doses of single-stranded nucleic acid (e.g. ASO) through the subcutaneous (s.c.), intravenous (i.v.), intraperitoneal (i.p.), intrathecal, intramuscular, or intracerebroventricular route. Mice will be euthanised after a suitable incubation period (2 weeks to 6 months).

The number of doses is the minimum to deliver sufficient compound (a single dose of double-stranded nucleic acid (e.g. siRNA) is adequate typically; up to 4 doses of single-stranded nucleic acid (e.g. ASO) may be the minimum necessary to attain sufficient levels to achieve the desired targeting effect). The routes of administration are the least invasive possible to achieve the necessary delivery (for example, where compounds administered s.c./i.p./i.v. do not reach the central nervous system in adequate quantities, this

necessitates intrathecal or intracerebroventricular administration). Wherever feasible, less invasive routes (s.c./i.p./i.v.) will be used.

Before planning studies with a given genetic target, we will scrutinise the literature for reports of effects on animals of genetic or molecular targeting of the same gene/protein, and of similar chemical structures being administered to mice. If targeting the gene/protein in different ways, or use of similar chemical structure and delivery route, is well-tolerated in the same species, it is highly unlikely that a nucleic acid compound will cause sequence-specific adverse effects.

Why can't you use animals that are less sentient?

The next less sentient viable animal model would be fish. These are not suitable for investigating organ distribution, since their organs are too different from humans (e.g. no lungs). Fish are also not suitable for investigating routes of administration, since many of these are not established or feasible in fish (e.g. intrathecal or intravenous injection). We cannot use mice under terminal anaesthesia due to the incubation times of 2 weeks minimum.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Comprehensive monitoring and welfare assessment (including Home Cage Monitoring Systems for new compounds), with use of analgesics or euthanasia if indicated

Minimize the dosing volumes Minimize the number of doses

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines

NC3Rs (National Centre for the Replacement, Refinement, and Reduction of Animals in Research) resources

LASA (Laboratory Animal Science Association) dosing guides

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

- Keep up to date with new literature in the field Attend international conferences
- Seek information on technical refinements from collaborators and colleagues Regular communication with local named persons
- Participate in discussions and attend seminars on 3R topics at the Harwell Research Campus
- Communication with the Medicines and Healthcare Regulatory Agency about optimal study design for translational projects aimed at bringing products to the therapeutic market

50. The evolution of the placenta across vertebrates

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

placenta, pregnancy, evolution, placenta development, pregnancy immunology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Poeciliidae	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how new organs originate and how they subsequently change in form and function across species using the placenta as a model. The placenta has evolved multiple times independently across vertebrates and we study its biology, development, and evolution in mammals and 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?

The placenta is responsible for transferring nutrients, oxygen, waste, and hormones between the mother and her embryo(s). Without the placenta, none of us would be here. Despite its crucial role, the placenta is one of the most poorly understood organs, and as a consequence, so are many pregnancy complications. An example is pre-eclampsia, a condition associated with maternal high blood pressure that can impact as many as 1 in 12 pregnancies and is a leading cause of maternal ill-health. This project aims to bring much-needed insight into the placenta, how it develops across different mammals, and how it

evolves across species. One of our focuses is on one of the most fascinating aspects of pregnancy - the mother's tolerance to the direct contact between her own cells and those of her foetus. We study how maternal immune systems have evolved different solutions in different mammals (including in humans) to deal with the challenges of the foetus on the mother's immune system.

The placenta is not, however, exclusive to mammals. Placentas have evolved more than 100 times independently across different vertebrates (for example, in some lizards, snakes, amphibians and fish). This makes the placenta an exceptional organ in which to study how organs originate and how they subsequently change in form and function across species. One family of small neotropical fishes (Poeciliidae) is exceptional from this point of view. Within this fish family (which includes guppies and mollies), placentas have evolved independently multiple times. We study this unique fish family to identify general principles (or rules) guiding the evolution of new organs.

What outputs do you think you will see at the end of this project?

There will be three main overarching outputs. One output will be a better understanding of the evolution of the mammalian placenta from a maternal perspective. Across mammals, maternal immune systems have evolved a variety of solutions to deal with the challenges of pregnancy. Identifying and understanding these solutions will shed new light on the evolution of the placenta. It will also contribute to our understanding of pregnancy complications, and it can potentially teach us about the immune rejection of transplants.

A second main output will be a description of how novel complex organs can evolve from scratch. Among Poeciliids, a placenta has evolved independently multiple times, and although sharing many features, the placentas from different species also show distinct morphologies. We aim to identify general principles underlying the formation of a new organ.

A third main output will be the identification of animal models that best mimic different aspects of the human placenta. Because the placentas of different species differ greatly, there is not a single best species that can serve as a proxy for the human placenta. By understanding the similarities and differences among placentas of different mammals, we will be able to identify the animal models that best resemble the human placenta.

We will share these outputs through publications in scientific journals and communications in scientific conferences and the press to quickly and freely disseminate our results to the general public and the scientific community.

We will also create a user-friendly (and freely available) database that compares placenta development across species. This database will allow the scientific community to access our data quickly and easily, maximizing its impact. It will also help in the choice of species and developmental stages for studying specific aspects of human placenta development.

Who or what will benefit from these outputs, and how?

The primary beneficiaries of these outputs will be other research groups that study the human placenta (in health and disease) and that of other species. We expect our novel findings to start being disseminated in scientific publications and conference communications two thirds into this project and continue for the ensuing years.

An output of this project will be a database, which will be available to all, describing (at the molecular level) placenta development across many species. These data will eliminate the need for smaller, targeted studies of individual species and/or developmental stages and serve as a resource for the scientific community. In the long run, it will reduce the number of animals used for these purposes. We anticipate making most of these data available before the completion of this project.

By comparing placenta development across multiple species, we will create a resource that will aid in choosing the most appropriate species to study different features of the human placenta. We anticipate that this will have a significant impact on the placenta biology community.

How will you look to maximise the outputs of this work?

We will create a database to make our data easily accessible to all researchers. We will produce the data in an unbiased and systematic manner to serve as a community resource. We expect our database to eliminate the need for smaller, targeted studies of individual species and developmental stages and hence, the number of animals involved.

We will disseminate the results from our work in multiple scientific publications. These peer-reviewed publications will be open access, that is, freely available to all. We will also disseminate our results through scientific conferences, national and international. To make our work accessible to the general public, we will write summaries for a lay audience and disseminate them in suitable outlets (general press).

This research involves collaborations with research groups from the UK, Europe, and the USA.

Species and numbers of animals expected to be used

- Mice: 4800
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The development of the placenta depends on an active collaboration between the mother and the embryo. There are currently no *in vitro* systems that can model this process properly. Our mammalian work will be primarily based on a collection of frozen tissues (placentas) obtained before the start of this project from several mammals (including humans, marmosets, mice, rats, rabbits, guinea pigs, horses, sheep, bats, and opossums). We acquired these frozen samples through multiple national and international collaborations. However, to study specific aspects of placenta development and function, we must work with genetically altered mice. We will work with two groups of genetically altered mice.

One group of mice have modifications that allow us to visualize under the microscope the cells where specific genes are active, which is key to understanding placenta

development. The other group comprises mice with genetic modifications that may impact the placenta, such as the maternal immune response to pregnancy. We are asking for permission to breed these mice in our animal facility to study their placentas. The placenta is an embryonic tissue that is only present before birth. Therefore, our work will focus on embryos and fetuses of both sexes and pregnant female adults.

The family of fish Poeciliidae (that includes guppies) is an exceptional model to study how placentas evolve from scratch because, within this family, placentas evolved independently multiple times. This means that we can study closely related species with and without a placenta, something not possible in mammals. Like in mammals, the fish placenta is an active collaboration between the mother and embryo and only exists before birth. Hence, our fish work will also be based on embryos of both sexes and adult pregnant females.

Typically, what will be done to an animal used in your project?

Mice: We are asking to breed genetically altered mice so that we can collect placental tissues from pregnant females. The tissues will only be collected after the animals are humanely killed using Schedule 1 approved methods.

Some mice will have genetic modifications that need to be activated by giving the pregnant female an activating substance. We will only use activating substances that do not produce adverse effects beyond their administration's mild and transient discomfort (e.g. oral gavage) or that cause adverse effects that can be counteracted by administrating other substances. For example, the adverse effects of tamoxifen, an activating substance, can be counteracted by the administration of progesterone. The activating substances will only be administered once per individual. We will collect placental tissues after the pregnant females are humanely killed using Schedule 1 approved methods.

Fish: We will humanely kill fish using approved methods. In addition to collecting placentas, we will also collect other organs/tissues (e.g. brain, ovary) to create a tissue biobank that we and others can use for future work, thus eliminating the need for further animal use.

Following veterinary advice, we may use hormones to induce some fish species to mate in case of low performance. The hormones will be added to the water in concentrations that will not adversely affect the fish. We will only use hormones if all other methods available fail (e.g. changes in nutrition, water temperature, environment enrichment) and if there are no known adverse effects associated with these hormones.

What are the expected impacts and/or adverse effects for the animals during your project?

Most genetic alterations in the mice that we will breed and maintain either have no impact on the animals or only have mild effects that do not impact their life quality in a laboratory setting.
Some genetic alterations may be associated with some embryo loss during early to mid-gestation or with difficulties in embryo implantation in the uterus. The latter is not expected to lead to adverse effects on the pregnant female, and the embryos are lost at a very early stage of development.

The administration of activating substances produces mild and transient discomfort. We will only use activating substances that do not have significant adverse effects or whose

adverse effects can be alleviated using other substances (e.g. progesterone in the case of tamoxifen).

No adverse effects are expected for the fish. We will only use hormones if they do not have side effects and are approved by the veterinarian.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of all protocols is set at mild, but the expected severity for the fish is sub-threshold.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The development of the placenta is a cooperative process between the embryo (from which the placenta originates) and the mother. There is a special tissue that forms during pregnancy in the uterus, called decidua, which works together with the placenta to provide the developing embryo with the nutrients and everything else it needs to grow. It is the placenta and decidua combined that is the functional unit that allows animals to grow in their mother's womb. Pregnancy is also a process that differs in many essential details between species. For both these reasons, we cannot study placenta development using *in vitro* (cell culture) systems. We also know so little about this system that we do not know how to approximate *in silico* these complex interactions between the mother and the embryo, and so no realistic *in silico* models exist yet (but we hope our research will ultimately help make these models possible). The placenta needs to be studied in its native complex context, and we can only understand the diversity of placenta forms and functions between species by studying those species.

Which non-animal alternatives did you consider for use in this project?
Because the development of the placenta depends on both the embryo and the mother and the complex interactions between them, it cannot be understood outside its native (animal) context. The existing *in vitro* models of embryo development do not include the placenta. Even if they did it, the critical maternal contribution, the decidua, would still be missing. However, we have tried to minimise as much as possible the use of animals. Most of this project is based on placenta tissue samples that we acquired through multiple national and international collaborations. However, to study some aspects of placenta development and function, we must work with genetically altered mice.

Why were they not suitable?

There are no feasible alternatives to studying the placenta outside its native (animal) context because we know too little about this system to approximate it using other ways. The development of the placenta is one of the most complex biological systems as it requires tight cooperation between two distinct individuals, the mother and the embryo. By studying the development of the placenta and its evolution across species, we aim to generate knowledge that will inform future studies and allow the development of *in vitro* and *in silico* models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We are aiming to breed 12 genetically altered strains of mice. We will breed these mice to maintain the strains in our animal facility, and we will set up specific matings within and between these strains to collect the placenta tissues (the pregnant females will be humanely killed). We estimated the total number of mice by multiplying the number of strains (12) by the number of mice that historically were required in projects with similar goals per year (80) times the 5 years (4800 mice).

We are aiming to maintain 10 species of Poeciliids. Of these, we estimate that we will need to administer hormones to promote matings to a maximum of 5 species. If we administer hormones to 40 females in each species per year, that will lead to 5 species x 40 females x 5 years, which is 1000.

We will also create a tissue biobank for the 10 species of Poeciliids. This means that in addition to collecting the maternal-fetal interface (i.e., the placenta in species that have this organ), we will also collect other tissues (e.g., brain tissues, kidneys, ovaries). Across the 10 species, we estimate that we will humanely kill and then harvest tissues for 500 individuals (50 per species) in order to cover both sexes and main developmental stages.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?
We established national and international collaborations to create a tissue biobank of hundreds of placentas from multiple species and developmental stages. The placentas were not collected specifically for this project. Through this step, we dramatically reduced the number of animals to be used in this project. We will keep using this strategy of collaborating with others to minimize animal use whenever possible. Additionally, to reduce the use of the Poeciliidae fish in future studies, we will collect tissues (in addition to the placenta) from humanely killed animals and create a tissue biobank.

We consulted with the staff at our animal facility, including veterinarians, to estimate the minimum number of animals needed to maintain genetically altered strains. We strive to use and maintain low numbers of animals by sharing lines and tissues from genetically altered mice with other labs and freezing embryos, tissues, and gametes whenever

possible. Because our work focuses on prenatal development, when we humanely kill a female mouse (or administer a substance), we can obtain tissues from the entire litter of embryos. Each embryo is a data point. Together, this strategy will reduce the overall number of mice used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whenever possible, we will obtain tissues (placentas) through collaborations. We have done this successfully for animals from multiple species that are not genetically altered, and we will aim to do the same for genetically altered mice strains. We will only breed them ourselves when this cannot be done through collaboration.

Breeding will be carefully controlled to avoid surplus animals, and when animals do have to be sacrificed, we will aim to collect as many tissues from as many animals as possible (after they are humanely killed). We will build tissue biobanks for the different strains that we and others can use in future work.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will work with two groups of genetically modified mice. One group of mice have modifications that need to be activated using gene activating substances (e.g. hormones). Some of these mice will allow us to visualise under the microscope (e.g., using fluorescence) the cells where specific genes are active, which is key to understanding placenta development and function. These genetic alterations do not cause any harm to the animals before and after activation. Other mice will allow us to understand the consequences of altering gene function in restricted physiological contexts, for example, in specific cell types and/or developmental stages. We will use these conditional alterations so that the modifications will only be active in a restricted group of cells and/or stages to minimise the negative effects on the animals. To activate the genetic alterations, we will give the animals the gene activating substances using methods (like oral gavage) that only cause mild discomfort and for a very limited time.

The other group comprises mice carrying genetic modifications with the potential to impact the development or function of the placenta, such as the maternal immune response to pregnancy. We will breed these mice and examine the placentas after the animals have been humanely killed. We can breed these mice because although the genetic alterations they carry may impact the placenta, they have restricted effects allowing for successful pregnancies.

For the fish species, the use of hormones to encourage breeding are not expected to have any negative impact, including pain or suffering.

Why can’t you use animals that are less sentient?

The mammalian placenta is an organ exclusive of mammals and can only be studied in mammalian species. However, because the placenta is only present before birth, most of the animals used will be at an embryonic or fetal stage of development. We will sacrifice the minimum number possible of pregnant females. Whenever possible, we will focus on embryos during the first two-thirds of gestation.

The Poeciliidae fish family is unique among animals because a placenta evolved independently nine times (for example, the mammalian placenta evolved only once, so all mammals share the same placenta). This means that uniquely in Poeciliids, we can compare multiple pairs of closely related species where one species has a placenta and the other does not. The Poeciliidae is a unique natural system that cannot be replicated with other species. We will study the fish before they are born, and we will sacrifice the minimum number possible of pregnant females.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The mice that we will breed will be monitored by highly trained animal technicians. There are ongoing efforts in our animal facility to test different bedding materials and shelters to identify those that maximise the animal's wellbeing. Different strains can have different preferences. We will apply the insights from these efforts to our mice to maximise their environmental enrichment. The mice that will be administered gene activating substances will undergo increased monitoring after the procedure. We will work together with fish specialists to maximise the environmental enrichment of our fish. If hormones need to be administered to encourage matings, we will precisely follow the instructions from the fish specialists and veterinarians and monitor these fish closely for the hours following the hormone administration.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow ARRIVE 2.0, PREPARE and NC3Rs guidelines. The NC3Rs monthly newsletter will keep us abreast of new developments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed through the NC3Rs monthly newsletter to which we are subscribers and through in-person training provided by our institution. Advances in the 3Rs are also shared internally at our institution via AWERB and NIO.

51. Towards a better understanding of pathological mechanisms of muscular dystrophy

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Duchenne muscular dystrophy, mdx mouse, purinergic signalling, inflammation, gene therapy

Animal types	Life stages
Mice	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 fully understand the pathological mechanisms behind the most common and highly debilitating muscular dystrophy and to use this knowledge to develop new and more effective therapies for this incurable 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?

Amongst over forty muscular dystrophies, Duchenne muscular dystrophy (DMD) is the most common and lethal inherited muscle disease. Affected boys demonstrate their first clinical symptoms in early childhood, develop progressive muscle weakness and wasting leading to a severe disability in their early teens and die at the age of 20-30. The physical symptoms can be exacerbated by a cognitive and behavioural impairment. Despite decades of research, no cure is available and treatments are symptomatic only. Recent data, including that from our laboratory, indicate that the understanding of the DMD pathology is incomplete and this hampers the development of effective therapies. It is

imperative that the disease processes are fully investigated, as the new knowledge gained should lead to more effective treatments.

What outputs do you think you will see at the end of this project?

New knowledge on molecular disease pathways will be obtained. In addition, mechanisms preventing and promoting dystrophic muscle regeneration will be described. This information will help to identify potential new therapeutic targets and also provide tools for monitoring the effectiveness of therapies. All the results will be published in medical journals to inform the researchers and clinicians, with whom we already collaborate. Key findings will be presented as press releases and information outlets aimed at the DMD community and the general public. Ultimately, some of these findings might lead to clinical trials and be translated into viable treatments.

Who or what will benefit from these outputs, and how?

Patients, patients' families and clinicians (and in the longer term the health care system) will benefit from the outputs generated under this project. In the short-term, findings released as quickly as possible shall lead to improved understanding of this pathology. Moreover, such findings should inform novel therapeutic approaches. Some of these, involving the re-purposing of existing medicines, could be implemented quickly and inform the course of this project. Completely novel treatments would be expected to enter clinical practice after completion of extensive clinical trials. Ultimately, the successful completion of this project should result in better treatments for this highly debilitating and ultimately lethal disease. Beyond the DMD, these findings may find application in other areas of biomedicine by increasing our knowledge of the basic biology of the cells involved.

How will you look to maximise the outputs of this work?

Our research is highly collaborative, both nationally and internationally, which leads to widespread dissemination of data, including the negative results (to avoid unnecessary repetitions), through collaborative meetings and seminars. Moreover, we attempt to publish all the results, whether proving or disproving the research hypotheses, in high impact scientific journals and present these at conferences. We make all our research data freely available to other researchers and value their scrutiny and critical comments. Furthermore, we work with parents' groups and keep them informed about any new and relevant developments.

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.

Explain why you are using these types of animals and your choice of life stages.
The mouse models of DMD are considered the most appropriate and efficacious for pre-clinical testing. Their use is the norm for making comparisons of the efficacy of any new method to previous approaches. We will be using two DMD mouse models, differing in the type of mutation and which we demonstrated to differ in specific aspects of the pathology.

Young adult mice, which show symptoms of the disease and also young pups to investigate the early, pre-symptomatic stages of this disease, will be used. Investigation of the latter life stage is important as we know that abnormalities are already occurring but the repair mechanisms are not fully operational yet. Therefore, the molecular pathways involved in damage and repair can be dissected. Furthermore, treatment at the early stages of this disease is expected to produce better results. Finally, older mice will be used to identify the long-lasting repair mechanisms. In addition to DMD mouse models, we will use mouse strains lacking specific proteins, which we discovered in earlier studies to be involved in the dystrophic pathology and which we aim to exploit as targets for developing novel, improved treatments. Genetically matched normal mouse strains will be used as controls in some experiments.

Typically, what will be done to an animal used in your project?

Mice will be bred to maintain the specific colonies and to generate new lines, which are dystrophic but lacking specific proteins that have been identified as important for the disease process. For breeding of genetically modified strains, the total of 5200 mice across five disease models will be used over the 5- year period. For the treatment, male mice will be handled, anaesthetised as appropriate, and the administration procedure undertaken. These could involve intramuscular or intraperitoneal injection or oral drug and/or gene therapeutic construct administration over a period of up to four weeks. For longer treatments drugs will be administered in drinking water, wherever possible, or implanted in a slow release form. 2000 mice will be used over the 5-year period. Some treated and control dystrophic mice will be tested for muscle strength and endurance and for their cognitive and behavioural functions using approved tests. 600 animals will be tested over the 5-year period. All animals will be killed at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

The DMD models to be used in this project show pathological symptoms akin to those in patients but these are transient (from 2 to 12 weeks of age), do not affect their mobility and do not lead to premature deaths. Mice genetically modified to lack specific proteins found by us to be involved in the dystrophic pathology have milder phenotypes than the original model and do not show any symptoms. Treated animals will experience a transient discomfort from the handling and the administration procedure. Bruising at the site of injection lasting for hours and exceptionally for a day may rarely occur. Weight loss associated with corticosteroid treatment may happen but will never be allowed to exceed 20% of body mass.

Expected severity categories and the 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 breeding of genetically modified mice severity is mild (5200 mice). The severity level of drug administration in drinking water is sub-threshold (50% of treated mice). The injection or oral drug administration and gene therapy treatments' severity level is mild (50% of treated mice).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are essential for this project as the dystrophic pathology results from a complex interplay between muscle degeneration and regeneration and immune and inflammatory responses, which contribute to both muscle damage and repair. Therefore, after completing all the possible in vitro tests, we cannot continue analysing these complex interactions and the efficacy of new treatments resulting from these new findings in an in vitro system. Tissue-specific ablation of the p2rx7 gene will explain the tissue- and cell-specific mechanism behind the therapeutic effect of p2rx7 ablation or inhibition that we previously identified. Moreover, we cannot study the functional efficacy of the purinergic blockade, which is under development as a DMD therapy as a result of our work under a previous licence, without pre-clinical testing in an animal model.

We have the zebrafish model of Duchenne muscular dystrophy (sapje) but while it replicates some of the key symptoms observed in patients and is being used by us for some applications. However, aspects of the fish disease relevant to this project are different and therefore the mouse models still need to be used.

Which non-animal alternatives did you consider for use in this project?

We use standard cell culture approaches, iPSCs differentiated into muscle cells, established muscle cell lines, muscle and macrophage cell co-culture system, and 3D muscle cell differentiation systems for some of our basic research.

Why were they not suitable?

The dystrophic pathology results from a complex interplay between muscle development and growth, muscle degeneration and regeneration and immune and inflammatory responses, which all contribute to both muscle damage and repair. The current co-culture systems cannot substitute for such a complex environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will keep the numbers of mice used to the minimum, consistent with the aims and the predicted numbers have been calculated based on our previous experiences with these specific experimental designs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In all our experimental designs we have been following the ARRIVE guidelines, as demonstrated in Sinadinos et al., 2015 and the TREAT-NMD guidelines (https://treat-nmd.org/research-overview/).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever feasible, material from individual animals will be used for multiple analyses e.g. serum for creatine kinase assays, diaphragms for organ bath analyses and leg muscles for other analyses. Much of the work will involve histological sections. These will allow for a number of different analyses to be made in each individual animal (e.g. muscle morphometry and immunological infiltration analyses).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

There are several mammalian models of DMD: mouse strains, two dog, one pig and one cat models. The characteristics of each phenotype and pathology have been analysed for pathological similarities to the human disease and costs of the maintenance. There is a consensus amongst experts that the most appropriate model to test efficacy for DMD are the mdx mouse and the golden retriever muscular dystrophy (GRMD) dog model. Mouse models replicate the symptoms of human disease between 2 and 12 weeks of age but these are transient, not reducing animal mobility and not causing premature deaths. This transitional nature will be exploited to identify mechanisms that limit disease progression, as these could potentially be exploited for the treatment of dystrophic patients. Moreover, mouse has been chosen as it allows comparing the results of medicines administration with those obtained in double knockout studies and to compare efficacy of new treatments to other therapeutic modalities already described using this model. All the treatments are tested in 2-12 week-old animals.
The tests to be used in this study are based on specific guidelines provided by TREAT-NMD (*https://treat-nmd.org/*), designed specifically to standardize experimental protocols that are used as efficacy readouts to allow comparisons of parallel efforts. Following an extensive consultation process TREAT-NMD identified a limited number of experimental protocols, which are appropriate for use in preclinical work and accelerate the development of new therapeutic modalities.

Importantly, in this study pain or distress are not a necessary concomitant to the validity of the experimental outcome. Therefore, animals will be observed and scored according to the checklist established in the initial experiment. Following administration, any animals

showing signs of unwanted effects other than those resulting from injection itself would be killed by Schedule 1 method and if multiple animals display such symptoms the experiment with the particular drug will be terminated.

Why can't you use animals that are less sentient?

We are using *sapje* dystrophic zebrafish, where possible. But this model does not reproduce the aspects of the human pathology that we wish to study under this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mouse colonies are monitored regularly for any signs of distress. Mice post-procedures involving anaesthesia are placed in the recovery chamber and monitored constantly until they fully recover. For the treadmill test animals are trained and the test is done under a constant supervision to prevent any signs of exhaustion.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animals in Science Regulation Unit (ASRU) Guidance and regulatory advice, TREAT-NMD for best practice in animal research in dystrophic conditions (https://treat-nmd.org/research-overview/preclinical- research/animal-model-choice-for-dmd/) and ARRIVE for publication guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular updates from the NACWO and NIO and attend refresher courses. In addition, web- based resources such as the NC3Rs (nc3rs.org.uk/), will be consulted. All the advances applicable to this project will be implemented immediately.

52. Novel therapies for inflammatory joint disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inflammation, Oxidative stress, Rheumatic disease, Arthritis, Therapeutics

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to improve our understanding of sterile inflammation and to develop new therapies for joint disease. Many joint diseases such as Rheumatoid arthritis, gout and osteoarthritis have a sterile inflammatory component that is an important part of the pathology. Inflammation is also closely associated with increases in oxidative stress and this work aims to develop novel therapies that target these responses as a means by which to treat these diseases. Osteoarthritis, in particular, is a growing problem worldwide as a result of the aging population so that improved treatments would contribute significantly to patient mortality and morbidity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 so that potential new therapeutics can be developed for the treatment of human diseases. In some cases (e.g. osteoarthritis) there is a significant unmet medical need for new treatments as there are no existing therapies that

prevent disease progression. The work described in this licence will also deepen our understanding of inflammatory mechanisms, how these are regulated in vivo and how they might be pharmacologically controlled. This research will also inform our understanding of how oxidative stress influences inflammatory responses with a view to developing new ways of treating inflammation.

What outputs do you think you will see at the end of this project?

Outputs from this project will include new information on novel candidate therapies for joint disease and better understanding of the relationship between inflammation and oxidative stress. This new information will be published in the academic literature. In the long term, the outputs of this project may contribute to the development of new treatments for joint disease.

Who or what will benefit from these outputs, and how?

The short-term benefits (3-5 years) of these outputs are for the scientific community, particularly those involved in the fields of inflammation and oxidative stress. In the longer term (5-10 years), the pharmaceutical industry may benefit from the knowledge gained by this work. In the very long term (10- 25 years), patients and the NHS may benefit from new and better treatments for joint disease.

How will you look to maximise the outputs of this work?

There are already a number of collaborations in place to ensure sharing of knowledge and experimental approaches. New knowledge will also be disseminated via publication in the academic literature. Information regarding unsuccessful approaches will be disseminated via publication and/or attendance at academic meetings.

Species and numbers of animals expected to be used

- Mice: 2100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 molecules tested in the animal models detailed in this project will have already been tested in cells in vitro and in ex vivo tissue and now need to be tested in vivo. This is particularly important as one of the major aims of this work is to test delivery of therapeutic molecules to sites of disease when injected systemically. This can only be tested using an in vivo model system.

Typically, what will be done to an animal used in your project?

Animals in this project will be treated to induce inflammation or a specific joint disease. Disease induction will be via injection of immunogenic antigens or via surgical procedure. Animals will then be treated with a therapeutic molecule which may be a protein or a nucleic acid. Treatment will usually consist of an injection administered intravenously or intraperitoneally and would normally be repeated three times per week. Some experiments

will be of short duration e.g. up to 48 hours while others will be much longer e.g. up to 20 weeks. The number of procedures that each animal will be subjected to will depend upon the particular protocol in use. Each protocol is related to a particular joint disease, with the exception of Protocol 1 which is designed to test the pharmacokinetics of new therapeutic molecules.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected adverse effects include discomfort or pain at site(s) of injection which is expected to be mild and transient (all protocols). In some cases, (Protocol 5) surgery may induce pain/discomfort which will be controlled by the use of analgesia. Some protocols will cause inflammation at specific sites (Protocols 3, 4 and 5) with moderate severity. The estimated duration of these effects is between 48 hours and 8 weeks depending on the experimental protocol. All animals will be humanely killed at the end of each experiment/protocol and every attempt will be made to set early humane end-points.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All protocols have a 'moderate' expected severity. Therefore, all animals under this licence are expected to experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The molecules tested in the animal models included in this project have already been tested in cells in vitro and via ex vivo tissue culture. These molecules now need to be tested for therapeutic effects in vivo. This is particularly important as one of the major aims of this work is to test delivery of therapeutic molecules to sites of disease when injected systemically. This can only be tested using an in vivo model system.
Which non-animal alternatives did you consider for use in this project?

All of the development and preliminary testing of anti-inflammatory molecules has been performed in vitro using cultured cells or ex vivo tissue. There are no non-animal alternatives that are appropriate for the next stage of testing of these molecules for therapeutic efficacy.

Why were they not suitable?

A major aim of this work is to test the delivery of molecules to sites of disease in the body

when administered systemically. Therefore, an in vivo system is essential and cannot be replicated using other approaches.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A statistician has been consulted so that the experiments designed have sufficient power to detect biological changes but will use the minimum numbers of animals per group. In addition, multiple parameters will be measured in each animal after death so that as much data as possible is obtained from each experiment thus avoiding the need to repeat experiments. This will not impact on the welfare of the animals while alive. Experimental bias will be reduced by use of randomisation when assigning animals to treatment groups and blinding of researchers to treatments received when analysing results.

I am wholly committed to publishing all data obtained in accordance with the NC3Rs ARRIVE Guidelines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animal usage will be reduced where possible. Some protocols have been specifically designed (Protocols 3 and 5) to induce disease in one limb so that the contralateral limb can be used as an internal control, so limiting the use of specific control animals. In order to reduce animal usage we will endeavour to gain as much information as possible from each experimental animal. Multiple samples will be collected when animals are terminated including blood, lymph nodes and inflamed tissue for fixation and subsequent histological examination. With Luminex and array technology it is now possible to sequentially measure levels of multiple mediators or their encoding RNA in small sample (50 µL) volumes through specific antibody or oligonucleotide interactions. This technology is available in our department and can further expand the information that can be assimilated from each animal where previously samples from several animals would have been required. The experimental designs detailed in this licence are based on the results of previous experiments performed previously in our lab and by our collaborators in other institutions. However, the NC3Rs Experimental Design Assistant will be routinely used to constantly monitor the numbers of animals used in our experiments based on new and emerging experimental data to ensure that animal numbers are reduced further if effect sizes allow. This will ensure that animal numbers are kept to the absolute minimum, while still obtaining robust scientific data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As mentioned above, wherever possible we will collect multiple data points from a single animal to increase data yield. Before any new study is conducted, we will conduct a range of in vitro and ex vivo experiments to enable us to provide reliable pilot data and thus allow

us to optimise dosing with test therapies. We will also make use of small-scale pilot studies in vivo to investigate observed effect sizes to determine whether an experiment can be completed with a reasonable sample size. If this is not the case, we will not proceed with that 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.

Each of the mouse models of disease detailed in this licence are well characterised in terms of their relevance to human disease. In addition, I have carefully considered the mouse strain used in each protocol so that the severity of the procedure can be minimised as much as possible. General measures taken to minimise welfare costs to animals include ensuring that all procedures are undertaken by suitably trained personnel and by ensuring that animals are inspected at least once daily during protocols so that unnecessary suffering is avoided. Pain relief will be provided where appropriate.

Animals will be caught prior to handling by methods such as catching and restraining using cupped hands or tunnels to decrease the stress and anxiety associated with catching mice by the tail.

Two protocols (Protocol 4 and Protocol 5) included in this licence are chronic in nature and painful and/or distressing for the mice. Therefore, there are specific refinements that will be applied when these protocols are used. The collagen-induced arthritis (CIA) model is useful for testing novel drug therapies because it mirrors the complexity of human RA in that both cellular and humoral immunity is invoked. At present, there is no spontaneous model in a GAA strain that resembles RA. The CIA model will be refined in a number of ways. To reduce the risk of ulceration at the site of injection of immunogen/adjuvant, a subcutaneous, rather than intradermal, injection will be given and the immunogen will be administered not at the base of the tail, but along the flank or back, where the skin is less prone to ulceration. Analgesics must be used with caution in animal models of arthritis because their anti-inflammatory activity tends to inhibit arthritis development in models of autoimmune arthritis.

However, dipyrone may be used as an analgesic as it does not inhibit disease progression or immune cell function when administered in the drinking water or by intraperitoneal injection. Analgesics will therefore routinely be administered prior to onset of arthritis and we will continue to optimise the dosing schedule and to evaluate alternative analgesics. Analgesics will be withheld if we suspect a pharmacological interaction with the drug under test. Additional husbandry support such as easier access to food (e.g. pellets placed at floor level) and supplemental bedding will be provided. General husbandry refinements for all protocols inducing chronic disease will include soft litter, nesting material, environmental enrichment and an appropriate number of cage-mates.

An important refinement is to limit the length of time that arthritis is present to the shortest possible time required to answer a given experimental question. Hence, mice with collagen-induced arthritis will normally be kept alive for a maximum of 21 days. For all dosing, the smallest needle diameter which allows rapid injection of the substance will be used, the smallest possible volume of substance will be administered and aseptic technique will be used. Where multiple injections are required, the most refined route of administration will be used, including slow release pellets or osmotic pumps. The use of humane endpoints, detailed in each protocol, will also ensure that no animal suffers unduly.

The model of osteoarthritis chosen is the destabilization of the medial meniscus (DMM) model that is the most refined of the OA models. Although this is a surgical model, it is minimally traumatic for the mice, leading to a slower progressing disease compared with those involving chemical induction or cruciate ligament transection. As described for the CIA model of arthritis, an important refinement is to limit the length of time that animals are in pain. The time-points chosen for analyses are 8 weeks, 12 and 20 weeks post-surgery to minimise the discomfort experienced by the mice while still achieving the scientific aims. Analgesia will also be administered just prior to surgery to minimise any pain induced by the DMM surgery.

Why can’t you use animals that are less sentient?

Experimental models of human disease are important for the development of new disease treatments. However, the challenge is to utilise experimental models that most closely mirror the human disease so that effective therapies in these models are likely to translate to novel treatments in the clinic. Mice are the lowest vertebrate group on which well-established models of disease have been developed. In this regard I will endeavour to refine our research strategy so that initial experiments are undertaken in the shorter models of disease and only the most effective therapies are examined in models requiring longer duration.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will continue to refine our methods to improve the well-being of animals, both by the updates provided by organisations such as LASA and NC3Rs and by contact with other research groups doing similar work.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to regularly update our knowledge on refinement using peer-reviewed, academic papers that use similar experimental methods. An important resource is the RSPCA resources on reducing severe suffering in animal research (https://science.rspca.org.uk/-/severe-suffering), with many of our refinements, particularly for the rheumatoid arthritis model, referenced on this site. We will also refer to resources, such as webinars and publications from organisations such as LASA and NC3Rs, including the LASA report on avoiding animal mortality, LASA guide to aseptic techniques, and NC3Rs newsletters.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular updates from The Laboratory Animal Science Association (LASA) and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). These organisations often provide educational material on how to improve animal well-being and minimise harm, and my laboratory members are encouraged to attend webinars hosted by NC3Rs. I also have regular meetings with my laboratory members to critically examine if any of these good practices can be adapted without altering our experimental outcomes.

53. Mechanisms and functions of sleep

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

sleep, torpor, brain, mice, hamsters

Animal types	Life stages
Mice	juvenile, adult, neonate, embryo, pregnant, aged
Siberian hamster	adult, juvenile, neonate, embryo, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Sleep is a vital process, necessary for well-being. The aim of this project is to obtain better understanding of the effects of sleep deprivation on the brain and the body, and of the benefits of sleep for metabolic regulation and cognitive functions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 have high impact for society, the economy and well-being because it will lead to a greater understanding of the mechanisms which regulate circadian entrainment, sleep/wake timing and sleep quality. Circadian rhythms and sleep/wake timing are

commonly disturbed by social and lifestyle factors, such as jet lag and shift work. Poor sleep is among the most prevalent complaints observed in many epidemiological studies, and the second most common overall complaint reported in primary care settings after pain. Disturbances in circadian rhythms and sleep/wake timing have a major impact on quality of life, resulting in impaired cognitive performance, increased risk of accidents as well as effects on immune function, hormone levels and cardiovascular performance. The amount of sleep and its quality also deteriorate with increasing age. As a result, elderly people are the main consumers of hypnotics, which have various side effects. A greater understanding of how circadian rhythms and sleep are disturbed by disease is necessary to enable therapeutic intervention. For example, data from this project are expected to provide a greater understanding of how sleep is affected by dysfunctional neurotransmission, typical for a range of neuropsychiatric disorders. In addition, there is a well-known trend for increased incidence of obesity, diabetes and metabolic dysregulation, which is often associated with reduced sleep quantity and quality, or disrupted wake/sleep patterns. Finally, this project will determine neurophysiological links between brain mechanisms underlying sleep and torpor. Inducing a reversible hypometabolism that mimics natural torpor in humans could have important influences on critical medical situations, including myocardial or cerebral ischemia, haemorrhagic shock, septicaemia, and organ transplantation.

What outputs do you think you will see at the end of this project?

The overarching aim of this project is to attain better understanding of the effects of sleep deprivation on the brain and the body, and of the benefits of sleep for metabolic regulation and cognitive functions.

This project will have high impact for society, the economy and well-being because it will lead to a greater understanding of the mechanisms which regulate circadian entrainment, sleep/wake timing and sleep quality.

Data from this project are expected to provide a greater understanding of how sleep is affected by dysfunctional neurotransmission, typical for a range of neuropsychiatric disorders.

Finally, this project will determine neurophysiological links between brain mechanisms underlying sleep and metabolism.

Who or what will benefit from these outputs, and how?

This project has academic and non-academic beneficiaries, including industry and patients. As our work will examine the fundamental mechanisms that underpin biological sleep need, this project will benefit a broad academic community. This includes basic and clinical researchers from the fields of neuroscience, sleep physiology, cognitive and circadian neurobiology, and neural engineering. The findings from our project will inform future studies addressing the effects of insufficient or disrupted sleep on brain function and bodily physiology, and will contribute to the development of novel approaches for sleep enhancement. The last years have seen a major increase in efforts to manipulate, or "enhance" sleep non-invasively and non-pharmacologically using direct or indirect (sensory) brain stimulation. Numerous companies spun out world-wide to develop these technologies, which attracted multimillion investments from venture capital firms and research funding. Our project will provide important experimental models and data that will inform future studies on sleep "enhancement". This project will also provide novel knowledge that will improve reproducibility of rodent studies, and will help to establish

novel refined rodent models for biomedical research. Our project will combine conventional transgenic approaches widely used in the field with a careful evaluation of the role of stress and environmental conditions, such as light. This will set the highest standards for sleep studies and will benefit numerous laboratories across the globe working on translational research.

How will you look to maximise the outputs of this work?

Outputs from this project will be maximised by collaborations and data sharing.

The protocols and electrophysiological data will be made openly available for access across the scientific community.

Al publications will be deposited on preprint servers and made open access upon acceptance.

Our work is regularly presented at national and international conferences, seminars and workshops.

Species and numbers of animals expected to be used

- Mice: 15000
- Other rodents: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Laboratory mice (Mus musculus) and Siberian hamsters (Phodopus sungorus) will be used in this project.

A part of the project will rely on mouse models, as this is the most commonly used species in sleep and circadian neurobiology, and techniques for chronic electrophysiological recordings, circadian phenotyping and transgenic manipulations have been well established in this species. These are also the phylogenetically lowest mammalian species commonly used in the laboratory with a brain sufficiently large to accommodate the recording electrodes required by this project.

Some experiments will be performed in Siberian hamsters. These animals show a pronounced seasonal physiology, including photoperiodism and photorefractoriness. We have established a successful breeding program for Siberian hamsters, which is designed to maintain winter phenotype in our hamster colony. When under short photoperiods, animals will be kept singly housed will be weighed and pelage colour will be scored every week. Animals that show high adaptation to short photoperiod after 8-10 weeks will be returned to the breeding protocol or included in torpor experiments. The brain size and anatomy of hamsters is similar to mice. The surgical procedures for implanting electrodes in Siberian hamsters are well established and will be similar to this procedure performed in mice. We will perform continuous sleep recordings in a subset of adult hamsters, where lighting schedules and ambient temperature will be varied to enable comparison with mice.

There is no alternative to using live hamsters in this project. Sleep and torpor are behavioural state and can only be defined and fully investigated in live animals.

Typically, what will be done to an animal used in your project?

Genetically altered mice and Siberian hamsters will be bred. They will undergo surgery to implant electrodes or probes that will allow us to monitor behaviour, body temperature or brain activity continuously. Mice maybe monitored in free moving or tethered conditions. They will also be singly housed during some of this monitoring.

The electrodes will also allow us to stimulate specific brain areas.

Mice may undergo chemical genetic alteration aimed at ablating or inducing genes of interest.

Animals may undergo sleep deprivation by being exposed to gentle stimulus or novel objects. We may also expose animals to altered light cycles to replicate those experiences that are like jet lag in the human. Behavioural tests will play an important part in our studies and animals will undergo habituation prior to testing. Some animals will under be exposed to periods of fasting or given restricted amounts of food or food at designated times rather than adlib.

The maximum duration of our experiment is 12 weeks but typically 3-6 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Weight loss (up to 15% decrease) for the duration of fasting/scheduled feeding single-housing typically for up to 12 weeks during sleep/circadian monitoring transient changes in the animals motor functions due to altered gene function.
transient discomfort and/or weight loss associated with gavage administration of substances local inflammation or post-surgical pain discomfort, minor surgical complications (transient).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: sub-threshold (50%), Mild (25%), Moderate (25%)

Hamsters: sub-threshold (50%), Mild (25%), Moderate (25%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Sleep, torpor and waking are complex behavioural states, which require use of live animals. Numerous brain areas are implicated in various aspects of memory, cognition and sleep-wake control and cannot be fully emulated in laboratory conditions or using computer modelling. The processes associated with cognition involve multiple distributed brain systems and therefore can also only be studied in alive freely-behaving conscious animals. Laboratory mice and Siberian hamsters are the best species for this project, as the techniques for chronic neuronal recordings and sleep studies have been well established in these species. These are also the phylogenetically lowest species of mammals commonly used in the laboratory with a brain sufficiently large enough to accommodate the recording electrodes as required by this project.

Which non-animal alternatives did you consider for use in this project?

Organotypic cultures, computer models.

Why were they not suitable?

Simple in vitro models do not allow the contribution of inputs originating from other brain areas to be investigated, which play a crucial role in the local and global neural dynamics during waking and sleep. The mechanisms of whole body metabolism can only be studied in vivo

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The determination of experimental and control groups in mouse and hamster experiments, numbers of animals and experimental design are based on extensive experience with the models

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A variety of measures will be used to reduce the number of animals for this project. Proper surgical procedures and postoperative care will result in better data quality thereby reducing the number of animals necessary to achieve the goals of this project. In order to reduce the total number of animals used to address the questions of this project, both mice and hamsters will be used, because depending on specific experimental series one or another species is more appropriate and suitable for obtaining reliable results using a minimal number of subjects. Specifically, some behavioural tasks, as well as pharmaco- and opto-genetic tools are better established and validated in mice, while hamsters are established species for investigating spontaneous seasonal torpor.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Careful colony management will reduce the number of animals necessary to achieve the research objectives.

We expect that not all animals will be able to complete some of the experimental series (e.g. some animals, not more than 20%, may be “slow learners” in the behavioural tasks). Those animals that will not reach the desirable performance level in the behavioural tasks will not undergo any invasive procedure and will be excluded from the experiment.

In some experiments, a conditional deletion of the gene of interest will be used by gavage administration of tamoxifen, which is known to produce more reliable results, and will therefore allow reducing the number of animals, which would undergo cranial surgery, behavioural tasks or other procedures.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All the procedures used in this project are well established and routinely used in the field. We routinely evaluate technical improvements to improve experimental conditions, specific procedures and animal welfare.

A part of the project will rely on mouse models, as this is the most commonly used species in sleep and circadian neurobiology, and techniques for chronic electrophysiological recordings and circadian phenotyping have been well established in this species.

Manipulation of the genetic status of mice by the administration of substances enables us to restrict the severity of those animals under the breeding programme. Most of our models are established or emerging models of neurological and neurodegenerative conditions such as Parkinson’s or Alzheimer disease and the use of genetic manipulation means they do not need to experience the phenotype unless it is required for an experiment.

Modern methods of mouse genetics enable us to selectively monitor and modulate the function of selected neuronal population in a highly refined spatio-temporal manner.

Surgical implantation of electrodes is necessary to record brain activity chronically in freely behaving rodents, and may require single-housing. However, the animals will be kept in pairs or groups for most of their lives providing social enrichment, and all singly-housed animals will have appropriate environmental enrichment provided, such as varied types of nesting materials.

Sleep deprivation is performed by the most ethologically relevant way, i.e. by providing naturalistic stimulation, instead of using long-term instrumental procedures, which appear to stress the animals.

Systemic injections, such as via subcutaneous, intraperitoneal, oral (gavage), intravenous routes, intraocular or with mini-pumps will be performed using the recommended route or that with minimum potential for adverse effects.

Appropriate choice of anaesthetic and analgesic regimes for pain relief will be used.

Why can't you use animals that are less sentient?

Using mammals is required for this objective because the anatomy of the brain and metabolic regulation in fish, amphibians and reptiles are significantly different from mammals

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For behavioural experiments, we have chosen appetitive tasks or investigation of spontaneous behaviours, which rely on a reward rather than aversive reinforcement.

Miniaturisation of implanted devices.

Monitoring of locomotor activity and body temperature, which is a gold-standard in circadian phenotyping and sleep research, is a non-invasive approach.

Carefully controlled housing conditions, proper surgical procedures and postoperative care, including pain management

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA guidelines, NC3Rs, ARRIVE guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By attendance at both internal and external NC3R's meetings. Attendance at internal animal welfare meetings where updates are provided by both the NC3R' regional manager and the Named Information Officer.

54. Elements that control the initiation and progression of fibrosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

transgenic, fibroblasts, LRP-1, cre-lox system, pathology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify the role of specific genes, proteins and regulatory elements within genes that initiate, maintain the progression of connective tissue fibrosis. We specifically wish to test gain and loss of function of these specific genes in lung and skin.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Fibrosis is defined as excessive tissue scarring, a common feature of most chronic diseases. There is very little in treatment for these diseases; most likely because each of these organs has a different purpose, is exposed to diverse environmental factors, and is composed of different cell types, there are unique features and consequences of tissue

fibrosis among these organs. We will focus our efforts on two organs: the skin and lung. We have previously shown that CCN2/CTGF and LRP-1 have a role to play in the pathogenesis of fibrosis. In addition, macrophages and epithelial basement membrane ECM-fibroblast cross talk have been shown to mediate part of this pathology. have been shown to mediate part of this pathology. It would be essential to characterise the mechanism by which they enforce their role. The benefit of this work is to understand better the pathophysiology of fibrosis and highlight molecules that can be used as a target to ameliorate the condition.

What outputs do you think you will see at the end of this project?

We hope to unravel the sequence of DNA that express during fibrosis and /or events that allow us to understand how the fibrotic process is initiated and maintained in skin and lung; especially, the role played by matricellular proteins, immune modulators and signalling molecules. The outcome will be communicated to the scientific community through publications and providing animal models of this pathology.

Who or what will benefit from these outputs, and how?

The ultimate beneficiaries are the animals and humans who succumb to this disease as we may provide targets for drugs that will either halt the initiation or progression of the disease state. Until then, the scientific community who are developing therapies will benefit, as well as pharmaceutical companies as they will be aware of the result of our investigation through publications and dissemination of our results.

How will you look to maximise the outputs of this work?

We will be using expertise in different areas: collaborating with clinical colleagues to interrogate the animal model as to the role of certain genes or molecules that are highlighted in human data.

Consolidate our research with similar models carried out elsewhere. To maximise the output, our work will be disseminated though our publications, press releases, national and international meetings to ensure we reach a wider audience.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are mainly using transgenic animals with cre-lox system in order to delete or overexpress the genes at the time we need. Mice are the smallest mammalian organism in which these technologies are available. The development of lung and skin are comparable to human tissues, and they breed in sufficient numbers to address funding cycles (mainly three years). We will use both embryos and adult mice to visualise the cells that are secreting excessive component of the extracellular matrix in the presence and absence of

the genes that this project is focusing on. The mechanics of the breathing cycles that inflate the lung and forces the expansion of the alveoli surrounded by the extracellular matrix that provide resistance cannot be replicated in less sentient animals.

Typically, what will be done to an animal used in your project?

In lung experiments: Single transgenic mouse carrying a floxed gene would be mated to a Cre mouse and double transgenics would be given a drug which will delete a gene during adulthood. The mouse will be administered bleomycin to induce lung fibrosis. mice will be monitored by in vivo µCT to analyse the level of fibrosis over three weeks and will be sacrificed at different intervals to harvest the lung for cellular and histological analysis.

In skin experiments: A mouse deleted of a specific gene may undergo punch biopsy of which will be monitored over two weeks. When a luciferase or fluorescent transgene is included, the mice will be imaged over time and then sacrificed and wound is compared with unaffected skin by various cellular, biochemical, histological and molecular means.

Similar treatment will be used with knock out or overexpressing mice to study the role of immune signalling in this process.

What are the expected impacts and/or adverse effects for the animals during your project?

In lung fibrosis, breathing distress can be seen with mice as they cannot cough but exhibit head twitching. The mice may refrain from activity about two days after bleomycin treatment. They will be weighed daily to monitor them. Normally they recover, if not they will be killed. Bleeding from the injury to skin, or extra scratching that prevent wound healing from occurring naturally. Skin irritation can occur in response to shaving or hair removal and animal may scratch the shaved skin.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity may be expected in 50% of mice that undergo lung fibrosis and 50% in skin injury.

Bleomycin induced lung fibrosis causes localised inflammation within the lungs and skin. At high dosage, bleomycin can cause breathing distress, the inflammation may exacerbate the condition, but the repair process normally subsides after initial treatment to be replaced by Extracellular Matrix deposition.

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?

Fibrotic process is multifactorial and involves a number of cells responding to each other as well as other insults. Neither cells nor ex-vivo can be used currently as a replacement. We need to use animals in order to understand the whole picture and mice in particular, since we can manipulate their genetics in a defined and control manner. The mechanics of the breathing cycles that inflate the lung and forces the expansion of the alveoli surrounded by the extracellular matrix that provide resistance cannot be replicated in cell cultures or less sentient animals.

Which non-animal alternatives did you consider for use in this project?

Whenever possible, experiments are conducted in cells first to check the system is working before we test them in animals. This includes defining DNA sequences as enhancers, working out the function of ADAM17 in macrophages, Cre-lox system, imaging of transgene in cells before we move to animal model.

Why were they not suitable?

The complex interaction between cells in an organ and those circulation from the blood such as macrophages in lung and skin cannot be replicated by one or two cell types. Therefore, we need a whole living organ to test the role of the genes involved.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Depending on the model, different number of mice may be needed. Our Null hypothesis= no change in ECM after injury. Alternative hypothesis: any change decrease or increase (Two tailed t-test). For comparison of skin treatment in terms of collagen change using two tailed- t-test on collagen changes; power calculation using our previous results suggests that with level of significance 0.05 and power 0.9 a std. dev. 0.43 we would need N=6 (G* power)

In repeated measurement, the level of significance >0.05 should be divided by the number of comparisons=3 (days after injury) This would increase the N=7.

A typical gain and loss of function experiment for pulmonary and dermal injury would include N=6 in each group;

Group1 sham PBS No Cre + TX Group 2 sham PBS +Cre +TX Group 3 injury No Cre- +TX Group 4 injury Cre- +TX

For one gene at three time points (1, 2 and 4 weeks)=72 x2 (gain and loss). Therefore, for 5 genes/ conditions =720 mice. Experiments may be repeated for intervention x2 =1440 animals

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental design allocated the right controls to make sure that the results are meaningful. In addition, introducing transgene that can be visualized such as luciferase or fluorescence will enable longitudinal study in the same animal rather than using more animals for the different end point.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The number of animals needed was based on power calculations as shown above based on published results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have refined two injury models over the last 5 years which are resulting in reproducible results. The bleomycin model in lung fibrosis which we identified the right concentration that does not reduce weight of the animals and not cause excessive fibrosis that causes severe adverse effects on tested animals.
Nonetheless allows us to monitor mice over four weeks. The other refinement is the wound repair or localised dermal fibrosis where we reduced the punches to two from 4 on each animal.

Why can’t you use animals that are less sentient?

Skin and lung are different structures and functions in mammalian system compared to fish for example or other less sentient. We need adult mice because we need to replicate lung and skin repair and diseases and therefore embryos are not suitable.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Over the last 5 years, we have already refined these two injury models.

We will always try to provide alternatives in route of administration of substances: for example, through food or drinking water rather than injection. Provide analgesic following surgery and increased monitoring and weight measurement to ensure animals are recovering. Terminate experiments when new unexpected phenotype occurs or consult local vet in case of scientific importance

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In the scientific community that work on animals , we all follow as the Animals (Scientific Procedures) Act 1986 (ASPA) and guidance. New development that focuses on " lessons learnt" disseminated by the NC3R website, LASA recommended maximum volume of administered substances and guidelines for blood removal as well as intervals between imaging sessions (LASA appendices A-C).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As an animal user, I currently receive updates from the NC3R through emails and the website and through the meetings held locally by the NC3R regional programme manager.

55. Mechanisms and treatments of craniofacial birth defects

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

craniofacial, cleft palate, craniosynostosis, treatment

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our research aims to improve the diagnosis, understanding and treatment of craniofacial disorders such as cleft palate and craniosynostosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Collectively, craniofacial abnormalities rank amongst the most common birth defects that may affect a new-born baby. These include cleft palate and premature fusion of the skull bones, which is known as craniosynostosis. Treatment of these disorders is a long term process and typically involves each child being cared for by a dedicated team of consultant

specialists including clinical genetics, radiology, dentistry, maxillo-facial surgery, anaesthesia, speech, language, hearing and psychological therapy.

The primary treatment for these defects involves complex surgical remodelling of the skull and facial deformities, which is aimed at protecting brain development and facilitating breathing, feeding, visual function, as well as the restoration of a normal craniofacial appearance. Although surgical correction is reliable, there is often a need for repeated surgical interventions from birth to maturity.

What outputs do you think you will see at the end of this project?

During the life of this project we expect to increase the diagnosis and understanding of the genetic, biological and molecular events that cause craniofacial disorders. This information will be published in peer-reviewed scientific journals so healthcare professionals can access this and inform patients and their families.

While the clinical application of a novel treatment may be some years into the future, by the end of this project we expect to have laid the groundwork for a new treatment of craniosynostosis that does not involve surgery.

Who or what will benefit from these outputs, and how?

New knowledge that improves our understanding of the diagnosis and molecular mechanisms of craniofacial disorders will be published in peer-reviewed scientific journals so healthcare professionals can access this and inform patients and their families. This will be possible within the lifetime of the project.

A potential non-surgical new treatment will have an enormous impact on the patient's quality of life and the development of the child as a whole, and on the patient's and families' quality of life. Full application of a new treatment into the clinical environment probably exceeds the life of the project.

How will you look to maximise the outputs of this work?

Our research is a collaborative project involving a multidisciplinary team of scientist and clinicians in the fields of mechanical engineering, developmental biology and craniofacial surgery.

Data produced as part of this project will be published in peer-reviewed journals, and where possible presented at (inter)national scientific meetings.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our research uses transgenic mouse models that mimic human craniofacial disorders. Mice are the animal of choice when studying genetic disease. Our work aims to understand the origins of the disease and develop ways to prevent or treat the disorder. For that purpose we mainly use embryos and pre-weaning animals when the disease is in it's early stages.

Typically, what will be done to an animal used in your project?

In this project, transgenic mice are used for breeding to create embryos or young animals (up to 21 days) for our biological studies. These studies mainly use post mortem tissues.

Adult animals (up to 3 months) are used for the development of a new treatment for craniosynostosis that replaces surgery. For this purpose animals undergo mechanical loading under anaesthesia. This involves applying a small load into a animal's skull bones for 10 minutes every day for two weeks.
Further analysis to assess the efficacy of the treatment is done post mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

Inherently, animals mimicking human craniofacial disorders will be adversely affected by the features of the disease (e.g. craniosynostosis). However, these animals are usually viable and breed normally, indicating a low level of severity. When in use, these animals will be carefully monitored by appropriately trained staff and specific husbandry methods will be applied to keep animals healthy.

Where we apply mechanical bone loading, animals will be under general anaesthesia. This is not because the procedure is painful, but because the animals have to stay still during the procedure. Previous studies have shown that animals recover quickly and do not show any adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Transgenic animals with a craniofacial disease are categorised as mild, with a small number (approximately 1%) showing moderate or severe features. Animals without a phenotype -including wild- type controls- are characterised as sub-threshold.

For the mechanical loading procedures, work under a previous licence has shown that no additional adverse effects are observed as a result of the loading and/or anaesthesia. Therefore the expected severity relates to the extend of the craniofacial phenotype in almost all cases.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Research into craniofacial birth defects concerns the mechanisms by which the head of an embryo/fetus develops its specific shape and associated function, and the disturbed mechanisms that underlie abnormal shape and function. Hence, an understanding of normal and abnormal development that leads to birth defects requires analysis of whole animals. While in silico modelling and in vitro tissue culture experiments can provide useful information, they cannot mimic the complexity of functioning organs in vivo, let alone the entire body.

Which non-animal alternatives did you consider for use in this project?

A possible alternative that was considered is the use of ex vivo tissue culture to replace in vivo experiments. Using this method allows the culturing of dissected bone tissue for a limited amount of time.

Why were they not suitable?

While this is suitable for a limited number of specific experiments, it does not allow analysis of the 3D structure of the skeletal system, in particular the skull. Also, it does not replicate the relationship between bone and the surrounding tissues e.g. muscle.

Importantly, the need for tissue does not negate the need for animals and there is a real risk of using more animals using a suboptimal experimental method.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 historic use of animals for similar projects and forward planning of future research projects.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Part of the research that is done outside of the scope of this license involves computer modelling of mechanical loading. This has allowed us to estimate the parameters for our in vivo experiments reducing the need to test a large range of experimental conditions using a (much) larger number of animals. Whenever possible, we use the NC3r's EDA to estimate numbers needed for experimental cohorts.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We aim to reduce the numbers of animals by using the most efficient and state-of-the-art breeding and husbandry methods available. We also include statistical power analyses to identify the minimum number of animals that we need in order to answer the research questions being posed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To achieve the research aims of this project we use genetically altered (GA) mice. Breeding of GA mice is performed to produce experimental animals needed for the study of the cellular and molecular mechanisms that underpin the relevant human diseases. Mechanical loading of bone tissue is employed to assess its potential as a novel treatment for human craniofacial disorders. Apart from breeding and mechanical bone loading, the large majority of experimental analysis is performed post mortem.

Why can’t you use animals that are less sentient?

Genetically altered mice enable the construction of animal models of human biology and/or disease that can be used in numerous ways to improve our understanding of disease processes and to develop new methods for diagnosis and approaches to therapy. Many studies of embryonic development employ sub-mammalian vertebrate or even invertebrate species. While each of these model systems has its advantages, the over-riding benefit of mouse studies is the relatively straight forward extrapolation of results to humans, and therefore to clinical disease. It is for this reason that genetic modification in mice forms the basis of this research programme.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We aim to minimise animal welfare costs by using the most efficient and state-of-the-art husbandry methods available during the breeding of GA mice. Colonies of GA mice are managed using good practise guidelines supplied by the NC3R and the The Jackson Laboratory. For mice with a craniofacial phenotype, increased monitoring is used to ensure animals are able to drink and feed as normal.
Sunflower seeds are used to enrich their diet and wooden blocks are used to manage any malocclusion.

We use bespoke equipment to minimise the welfare costs associated with procedures requiring the in vivo testing of novel treatment protocols. A 3D printed nose cone is used to

allow mechanical loading of the skull while using inhalation anaesthesia, avoiding the need for injectable agents and/or head restraint.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will employ the good practise guidelines provided by LASA and the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will employ the good practise guidelines provided by LASA and the NC3Rs and ensure these are up-to-date at all time by regularly checking their websites and signing up to relevant newsletters. We also stay abreast of the scientific literature concerning the 3Rs.

56. Visual function and gene therapy in 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Retina, Gene therapy, Vision, Eye

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 better understand how the eye processes visual information and how different genetic and degenerative conditions affect this. A major focus of the project will be to evaluating the safety and success of new gene therapies and will also aim to solve some of the technical limitations currently facing this therapeutic approach

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 aspects of visual function that we still do not understand, including how particular genes define the function of the different cell types within the retina. The

outcomes of the project will enhance our understanding of how the visual system works and in particular will deepen our understanding of pathological conditions that cause partial or total loss of sight. For the majority of conditions that result in blindness no treatments are available to halt the progression of the disease or to improve vision. The work outlined in this project will support the development of new treatments that will aimto prevent or reverse blindness.

What outputs do you think you will see at the end of this project?

The work of this project is expected to advance the scientific understanding of how the retina process light information, particularly which cells and what mechanisms are important for specific aspects of visual function. It is also likely to advance our understanding of how particular genetic conditions affect vision and why, with an emphasis on identifying which molecules are linked to particular genes and which cells need these molecules to function properly. By the end of the project we should have identified new candidate molecules to improve vision or prevent blindness in several eye disorders, including diseases caused by genetic conditions as well as visual problems associated with aging or physical damage. The research will also pioneer new technologies that aim to overcome the current technological limitations of gene therapy that may have broader applications in other fields of medicine.

Who or what will benefit from these outputs, and how?

The work of the project will benefit the wider research community particularly those working in the fields of visual neuroscience, genetics and gene therapy, by providing new insights into the physiology of visual function and development of new technology to treat genetic conditions.

Patients with genetic and degenerative conditions affecting visual function will benefit from this research through gaining a better understanding of the pathology of their disease, potentially including improved diagnosis and more accurate prognosis. They also stand to benefit from the development of new therapies that will be able to improve their visual function and/or slow or prevent disease progression.

How will you look to maximise the outputs of this work?

Findings from the project will be regularly disseminated in internal seminars, inviting insight from a broad range of scientific disciplines. The work will be presented more formally at national and international conferences and published in peer reviewed, open access scientific journals when appropriate. New intellectual property arising from the findings will be identified and appropriately protected by the filing of patents promoting the advanced development of these findings for translation to the clinic.

Species and numbers of animals expected to be used

- Mice: 3300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All experiments for this project will be performed in mice. Both in-bred strains of “wild-type” mice and genetically altered strains will be used. Many genetic strains are matched genetic models for the genetic conditions that cause retinal dystrophy in human patients. This makes them very valuable models to study the physiological and pathological consequences of the disease and to accurately evaluate the potential for a new therapeutic intervention

Typically, mice will be used in these protocols post weaning age for 2-6 months. This is to allow the visual circuitry to fully develop and model "adult" presentation of ocular diseases.

For some aspects of the project mice will be used at pre-weaning age (P0-P21). This is to allow measurement of aspects of the disease that are apparent in early development. Treatment with novel therapeutics may also be trialed in pre-weaning age mice to evaluate the importance of therapeutic intervention at early stages of disease progression.

Typically, what will be done to an animal used in your project?

Typically mice used in this project license will undergo the following procedures:

Mice will undergo behavioural monitoring in specialised experimental set ups that allow their responses to visual cues to be measured.

Whole eye responses to light stimuli will be recorded using electrodes placed on the surface of the eye. These experiments are performed under general anesthesia for the purpose of restraint. These measurements will typically be performed before and after therapeutic intervention, or at different time points during the lifespan of an individual mouse, to measure changes in visual function during development of the disease.

Experimental animals will receive injection of experimental or sham substances into the eye under general anesthesia for the purpose of evaluating the safety and/or efficacy of novel therapeutic agents.

At the end of all in-vivo studies, mice will be humanely killed. Tissue (particularly from the eye) will be taken for further study. Study duration may be from several weeks up to 2 years.

What are the expected impacts and/or adverse effects for the animals during your project?

- Mice will be purposefully bred with genetic conditions that cause poor quality vision or blindness. This will cause some degree of sensory deprivation for part or all of the lifespan of the animal.

- Behavioural monitoring and measurements are not expected to cause adverse effects or distress.

- Repeated general anesthesia required for measuring electrical responses of the eye has the potential to cause adverse effects. Experiments will be designed to minimise the duration and number of procedures carried out under general anaesthesia. Mice will be carefully monitored during their recovery.

- Ocular injection procedures can cause localised trauma to the eye (for example small

retinal tears, retinal detachment, localised bleeding) and/or transient increased pressure within the eye. These issues typically resolve quickly and spontaneously within a day or so. Occasionally the injection can cause localised inflammation, which would lead to some discomfort. This is usually avoided by the application of antibiotic ointment at the end of the injection procedure. Injected mice will be checked daily until all adverse signs of the injection have resolved.

- The introduction of novel experimental or therapeutic genes is designed to alter the function of specific cells within the retina. They are either not expected to cause changes to visual perception, or only slight to modest changes to visual perception, and are not expected to cause any discomfort. However mice will be regularly monitored by observation in the home cage to look for signs of visual disturbance that might be causing distress (for example, excessive eye closure when awake).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Overall for this project 60% of mice the expected level of severity Moderate and for 40% of mice it is Mild.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project is aiming to look at the very fine detail of how biological systems are able to process visual information. This requires that the cells and circuits develop normally as part of an entire biological system. Similarly, the response to therapeutic intervention needs to be assessed in an intact biological system if it is to be developed for use in human patients. This allows more accurate prediction of the therapeutic effect as well as allowing for observation of important adverse effects.

Which non-animal alternatives did you consider for use in this project?
Considered alternatives included 2D and 3D (organoid) cell cultures, computer modelling and studies performed in humans.

Why were they not suitable?

Cell culture

Cell cultures are unable to replicate entire biological system, including all the cells types, their neurological connectivity, blood supply and immune response. These are important aspects to model if the aim is to understand how biology creates visual perception, the changes that occur in disease and critical assessment of new therapeutic targets. However, cell cultures and organoids will be used to fulfil the overall scientific goals of the program of work. They will be used to directly replace animal experiments wherever possible (for example in screening vector serotypes specificity) and they will be used in parallel to animal studies to enchance the general scientific conclusions and/or statistical power of study outcomes through meta-analysis whenever possible.

Computer modelling

Not enough is known about the biology, the connections between cells and visual function to be able to model diseases accurately enough to use computer models to answer scientific questions. However, computor models will be routinely used in the program of work to refine research questions, aid design of experiments and increase the statistical power of study outputs (for example by using bootstrapping methods to predict or model variability).

Studies in humans

The methods that will be used to study the how the biology functions, for example through introduction of experimental substances that alter the function of retinal cells, are too invasive to undertake in human subjects. However non-invasive studies in volunteer human subjects and patients (for example psychophysical measurements and electroretinograms) will be undertaken in parallel throughout the program of work to continually refine research questions, maintain translational relevance and to enhance the scientific conclusions through a multidisciplinary approach.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimation is based on previous knowledge and experience working in the field of visual neuroscience and gene therapy. Historical and published data for all of the techniques and some of the specific genetic strains named in this project provide an estimation of variance for many planned experiments. This allows the number of animals providing results with a clear interpretation to be predicted using statistical power calculations.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

General considerations

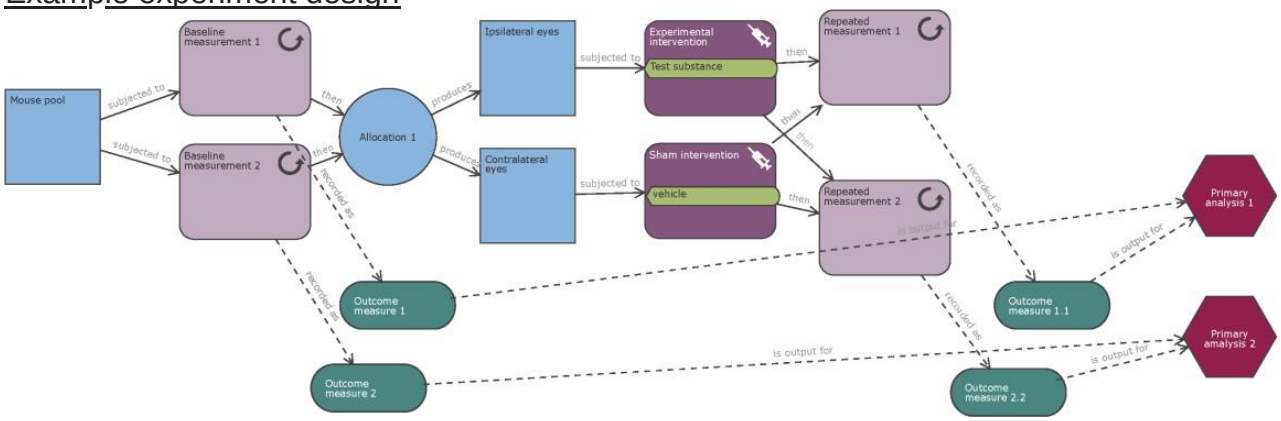
- The use of power calculations was used to predict sufficient sample size.

- Incorporation of block randomisation of treatments (to left or right eye, and/or across animals housed in the same cage) will be used to maximise the statistical power of each animal used in each study.

- Randomisation of left to right eye will be used wherever possible to provide internal controls and maximise the statistical power of each animal used in each study.

- Meta-analysis will be incorporated into the analysis where multiple measurements (for example behavioural measurement and electro-retinograms) are performed within single animals to maximise the statistical power of each animal used in each study. This can be done whenever each outcome can be used independently to support or reject a hypothesis.

Example experiment design



What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Historical and published data will be used to guide the design of new experiments. Where historical data for particular models or techniques is unavailable pilot studies will be undertaken to explore unforeseen technical challenges or variability before expanding to use on larger groups of experimental animals.

Throughout the duration of the project the experiments carried out in animals will be complimented by work done with computer models, human subjects and 3D tissue cultures made from human stem cells. These alternative methodologies will be used to undertake any related experimental work for which invasive techniques and/or intact biological systems are not required. For example, tissue cultures will be used screen the potential suitability of new therapies before using them in animals (for example screening vector serotypes). They will also be used to define and refine research questions. For example, studies measuring patient vision or predictions made with a computer model can be used to decide which specific aspects of retinal function need to be investigated further in an equivalent genetic mouse model of the disease. Computer modelling can be used to more accurately predict variability from pilot data using bootstrapping methods, enhancing the use of power calculations. Wherever possible statistical meta-analysis will be used to analyse outcomes from related studies within the multidisciplinary 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Both in-bred strains of “wild-type” mice and genetically altered strains will be used. Many genetic strains are matched genetic models for the genetic conditions that cause retinal dystrophy in human patients. Specific strains may include Cnga3-/-, Pde6c-/-, Abca4-/-, Cep290rd16, Gnat1-/-, Pde6b-/- mice. This The match with human genetic condition makes them very valuable models to study the physiological and pathological consequences of the disease and to accurately evaluate the potential for a new therapeutic intervention. A laser induced model of focal photoreceptor loss in C57 wild-type mice will also be used to model the pathophysiology of macular degeneration in humans. The general approach of these studies center on performing comparative physiological and behavioural studies in genetically modified mice that closely model the pathological condition in patients. Techniques include electrophysiological measurements of the retina (ERGs) and measurement of visually guided behaviours. Interventional approaches, such as AAV-based gene therapy, will be used to try to alter the pathophysiology of disease progression. Important techniques for these approaches include ocular injection techniques.

Why can’t you use animals that are less sentient?

Mice are the lowest order vertebrate that has an adult retinal structure similar to humans, comprising many of the same cells types, structural features and cell functions. The genetic mouse models to be used in the project are an exact match for the genetic problems causing blindness in humans. This ensures that the findings from this research will fulfil the objectives of the project; advancing our knowledge of how the retina processes information and progressing the development of new therapies to cure blindness.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will be continually reviewed for possible refinement. A recent refinement include changing from vision based operant conditioning tasks to a low-severity behavioural assessment paradigm based on instinctive responses to naturalistic visual cues.

Advice from the NACWO and NVS will be regularly sought in particular when implementing new techniques.

Highly experienced investigators will carry out and/or supervise surgical procedures and physiological measurements.

Mice will be housed with several cage-mates unless mice need to separated for specific reasons, (e.g. in-cage fighting). When used, periods of single housing will be kept brief (up to 48h) before attempting reintroduction to cage mates.

Mice will be provided with quality bedding material, with particular consideration of

minimizing eye irritation (i.e. moderate chip size, dust free). Mice will also be provided with home cage enrichment.

Peri-operative monitoring and analgesia will be used for as long as necessary following procedures to ensure minimal suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be performed in keeping with best practice guidelines (LASA Guiding Principles for Preparing and Undertaking Aseptic Surgery)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute has a NC3Rs advocate who regularly disseminates new information about experiment design, methods and replacement technologies to all investigators involved in animal research. The scientific literature is continuously scrutinised and discussed by academics at the institute through informal lab meetings, journal clubs and seminars. We will strive to identify and implement new techniques that could promote the 3Rs.

57. Poultry welfare: effects of housing and management

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

poultry, housing, management, beak trim, disease detection

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to improve the welfare of poultry by studying their housing and management conditions (or some aspect affected by these conditions, such as disease). There are 3 main sub-aims to address this:

Aim 1: Establishing how poultry can be housed and managed without beak trimming. Birds will be kept in one or more housing systems and at stocking densities that may reflect commercial practice so that the results are likely to translate to industry. Because bird strain has been shown to affect the results, we will use strains that are used commonly in the UK and the EU, and/or which show differences in injurious pecking behaviour, and/or which have been shown to differ in beak shapes due to their beak or bone morphology. Birds may be left with intact beaks, or may be compared to birds that are beak trimmed using one or more commercial methods, in order to establish if birds can be housed without beak trimming (e.g. without injurious pecking or pecking-related mortality being much higher than birds that are beak trimmed). Trimming methods and age at application will follow common agricultural practice to make the results relevant to the industry. Birds may be exposed to mildly aversive conditions (e.g. lack of environmental enrichment), since pecking behaviour is known to be exacerbated by stress in commercial environments, which we would aim to mimic. Birds may be given access to novel

enrichment (e.g. hay bales, string) to help reduce injurious pecking, which might alleviate the need to beak trim. Birds may undergo pecking tests, whereby the pecking damage they inflict on an inanimate model is measured. The scientific outputs of these studies will be the observations of differences in e.g. behaviour, injurious pecking rates and/or feather pecking damage, body weights, feed intake, or physiological signs of stress measured via blood sampling. This information will help elucidate ways to house these types of poultry without the need to beak trim while still safeguarding their welfare.

Aim 2: Determining production and welfare levels for different strains of broilers from hatch through to rearing, catching, transport, and slaughter (including meat quality). Broilers will be housed in styles that mimic commercial practice including for such schemes as the RSPCA Assured Scheme guidelines (https://business.rspcaassured.org.uk/media/qbuhlsvd/meat-chickens-standards-rspca.pdf), so that the results are more likely to reflect industry practice. Birds will be reared in litter-floor pens from hatch and video recorded for behaviour throughout the trial. Birds will be fed nutritionally- appropriate diets and the feed consumed will be recorded. Bulk and/or individual weights of the birds will be taken at regular intervals, as will welfare quality measures such as hock scores, foot pad scores, and/or gait scores. At the end of the growing period, birds will either be culled directly at the PEL for tissue/meat quality data, or after a period of transport to a processing plant. To mimic commercial transport conditions, birds may be loaded into transport crates and may either be a) driven along a prescribed loop that begins and ends at the PEL, with bird inspections carried out every time the PEL is reached, with the final journey ending at a processing plant or back at the PEL; or b) driven directly to a processing plant. At the processing plant, birds will be killed using commercial methods and meat quality measures recorded. This information will help us determine production (e.g. weight gain, feed consumed, meat quality) and welfare (e.g. foot pad score, gait score) measures for each strain of bird which will identify which birds can be used in higher welfare schemes. It will also allow for determination of which strains of birds can be used in production while potentially having better welfare than other strains. The novel strains will be compared to known established slow-growing broiler strains. The scientific outputs of these studies will be welfare quality measures such as footpad and hock scores, lameness scores, cleanliness scores and time spent in various behaviours, as well as production measures such as bird weight and feed conversion ratios. This information will help us determine the levels of welfare for different strains of broilers and assess which strains could be used in higher welfare schemes which could encourage more producers to become involved in these schemes.

Aim 3: Using technologies to detect early signs of disease. Laying hens will be housed in styles that mimic commercial practice (e.g. litter floor pens or enriched cages), and will be fitted with automatic recording equipment (i.e. sensor technology) suitable to their size and species (such as accelerometers and/or tags that track their location). Hen activity from the sensors plus recordings of hen behaviour from live observations or video recordings will be measured concurrently. Hens will be assessed before and after a vaccine challenge to stimulate changes in behaviour that mimic sickness behaviour. In order to reliably induce sickness behaviour using vaccines, the hens may be previously unvaccinated. The aim is to see if the sensor technologies can identify these 'sickness behaviours' in birds, which could be used in commercial systems as early warning indicators. This is a particularly important technological advancement that could be used in large (commercial) flocks, where stock worker observations alone may not be adequate to identify early changes in flock behaviour due to illness. This will contribute to good management of flocks.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Hens and broilers are the workhorses of agriculture: in 2019, there were approximately 25.9 billion chickens in the world (statista.com), mostly reared to feed people via eggs and meat. As a consequence of being domesticated farm animals that are housed for much, if not all, of their lives, chickens rely on the ways that they are housed and managed by us for a good-quality life. The work described here focuses on housing and management methods to improve the welfare of chickens. This work aims to look at solutions to common problems experienced by hens and broilers due to the way they are kept or managed, such as beak trimming, disease, and rapid growth rates, that are practical (i.e. able to be implemented at farm level) while being sensitive to the needs of chickens.

What outputs do you think you will see at the end of this project?

For laying hens (beak trimming): the work will generate information on which commercial housing methods, used with or without additional enrichment, and/or which hen strains or types of beak morphologies can be used without the need to beak trim.

For broilers: the work will provide evidence of what strains of birds can meet production targets while having improved welfare over conventional fast-growing strains.

For laying hens (disease detection): this work will identify useable technologies that help producers detect the early signs of disease in large poultry flocks, which can improve the success of treatment and/or limit the spread of disease to other flocks. In all cases, outputs would include scientific publications, trade articles, and conference presentations.

Who or what will benefit from these outputs, and how?

For all areas investigated, beneficiaries will include commercial poultry, poultry breeders, poultry farmers, consumers, and fellow scientists. These are unlikely to be fully realised until each project is completed (up to 2 years after the completion of this licence).

How will you look to maximise the outputs of this work?

Broilers: work is typically done in collaboration with breeding companies and animal welfare and/or accreditation schemes, thus outputs are disseminated directly to the place of maximum impact; outputs will also be disseminated in peer-reviewed journals and technical (trade) articles.

Laying hens (beak trim): work will be done in collaboration with a major laying hen breeding company, and will make use of their existing genetic database and parent flocks; outputs will be disseminated in peer-reviewed journals and technical (trade) articles.

Laying hens (disease detection): work will be done as part of a PhD project, whereby the student is required to publish his work in peer-reviewed journals. The work will also be presented at scientific conferences and disseminated to the technology producers.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 1800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Studying the pros and cons of housing and management techniques for hens and broilers relies on the response of the whole animal in order to judge what is beneficial (or not) to their behaviour, health and welfare. All animal types and life stages are chosen on the basis of relevance to commercial practice. Broilers are typically housed as juveniles (from day old up to 12 weeks), because this is the period of growth to slaughter, whereas hens are typically studied as adults (16 weeks onwards) as this is when they are producing eggs, but may also be studied as juveniles, since this influences well-being as adults. Particularly where we want to study sickness behaviour in hens, we may use non-vaccinated pullets (i.e. from hatch to sexual maturity) for later use in those experiments.

Typically, what will be done to an animal used in your project?

Birds will often be housed in commercially-relevant housing (in terms of floor surface, available furniture, and stocking density). Some birds may undergo blood sampling to measure their physiological responses. Some birds will not be beak trimmed and therefore may be at greater risk of damage from feather pecking and cannibalism, if they develop. Some birds will be given a vaccine to stimulate a short-term sickness response so that we can measure if novel technologies can identify sickness behaviour. Experiments typically last for several weeks or months, in order to study long-term effects on behaviour.

What are the expected impacts and/or adverse effects for the animals during your project?

Long-term impacts may come from living in commercial-style poultry housing, such as weakened bones (hens in enriched cages cf. floor systems) or greater keel bone damage (floor systems cf. enriched caged), or foot pad disorders (broilers on litter at commercial stocking density). With beak trimming, long-term pain and lasting harm are potentially more likely to occur due to *not* applying this commercial treatment, if damaging pecking behaviour develops. For birds undergoing beak treatment, the effects have been shown to have short-term effects on beak related behaviours (i.e. days). Birds challenged with a vaccine are expected to show short-term sickness behaviour; however, this is expected to be transient and the work proposed only uses poultry-approved products; therefore this is not outside the experience of poultry normally. Sickness-behaviour studies may use previously non-vaccinated poultry, to more reliably generate a response.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: 80%

Moderate: 20%

(Calculation: Protocol 1 (15% of 100 birds moderate) + Protocol 2 (15% of 1600 birds moderate) +
Protocol 3 (100% of 100 birds moderate) = 355/1800 moderate = 20%)

What will happen to animals at the end of this project?

- Rehomed
- 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?

Behaviour and welfare studies need to study the whole animal and their responses to their environment.

Which non-animal alternatives did you consider for use in this project?

For studying pecking behaviour in different strains of hens with different beak shapes, we are using models for hens to peck at (rather than pecking at other birds). While we understand that models (e.g. letting hens peck at agar gels, or dummies covered in chicken skin) will not reflect the exact damage that a live bird would receive, they will allow us to get a relative measure of different damages inflicted by different beak shapes.

Why were they not suitable?

In other studies in this licence, there are no models that can encompass the complex multi-system mental and physical activities of the whole animal to give accurate responses to the measure we want to undertake.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on the types of projects we will undertake and the related numbers of treatments and replicates per experiment, per project:

Currently, we do trials looking at broiler breeds without the need to run the study under license, and in those trials, we use 1600 broilers

Plans for studying beak treatment and beak shape amount to no more than 100 hens

Plans for studying the use of sensors to detect disease amount to no more than 100 hens

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We base our animal numbers on prior experience and after discussion with a statistician, to ensure that the number of animals used is neither too high nor too low. We use optimal experimental designs and analyses that take account of all sources of variation, to get the maximum information from our studies. The use of simulated commercial conditions in some studies impacts the number of animals used (e.g. they are typically higher than laboratory-based studies), however this means that the relevance of our work to the agricultural industry is likely to be greater.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whenever possible, we share data from our studies with students (BSc and MSc level) for dissertation projects.

Where animals must be killed for tissue collection, we will offer extra tissues to colleagues in the local area for use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Chickens are used because their housing and management systems, and consequences of farming (and how the chickens respond) are the areas of interest.

Why can’t you use animals that are less sentient?

In order to reflect on commercial farming of animals, it is important to use the species used by the farming community to get accurate data.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

With every experiment performed, we carry out a harms/benefits analysis, and review these retrospectively to see how measures can be refined for future similar trials. These are the requirements of our AWERB. This assists us in planning future trials to minimise welfare costs to animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Various guidance provided at the NC3Rs website.

Home Office Code of Practice on non-human primates, farm animals and birds (Dec 2014).

We also use ARRIVE guidelines for publishing research appropriately, so that other researchers can refine their techniques accordingly.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We share knowledge on best practices with other research groups doing similar studies. I am in receipt of monthly email updates from NC3Rs about their events and publications.

58. Improving the understanding and treatment of Parkinson's disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Parkinson's, Therapy, Motor symptoms, Non-motor symptoms, Neurodegeneration

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to improve our understanding of the processes that underpin Parkinson's disease and to find better treatments for this debilitating, chronic condition.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are currently over 6 million people living with Parkinson's disease and in 2016 alone PD caused over 211000 deaths. Prevalence rates have increased by 22% in the past thirty years alone, making Parkinson's one of the fastest growing neurodegenerative disorders. Parkinson's affects motor coordination in individuals but triggers many other non-motor symptoms too, including anxiety, pain, sleep disorders, cognitive dysfunction and bladder dysfunction, to name just a few. Drugs are available to bring some relief of the motor

symptoms. However, the gold standard drug, levodopa, causes side- effects in many people that invoke uncontrolled, excessive movements so is less than ideal. Moreover, the underlying causes of some of the symptoms in Parkinson's, such as pain, are also poorly understood and so their management with existing drugs is also currently inadequate. Finally, there are no drugs currently available that slow down or better still stop the progressive death of the vulnerable brain cells which drive the ever-worsening symptoms. The work undertaken in this project will explore new avenues for discovering drugs that can bring much-need protection or repair to the affected brain cells. In addition, by exploring some of the non-motor symptoms such as pain and cognitive dysfunction, as well as those underlying levodopa-induced excessive movements we will increase our understanding of the causes behind these symptoms and side-effects, respectively, and therefore help to unveil new treatment strategies.

What outputs do you think you will see at the end of this project?

This project will provide new information on therapeutic approaches that could lead to drug treatments to slow down the progression of Parkinson's or repair existing damage in the brain post diagnosis.

There is potential for some of these approaches to progress into clinical trials, if successful in our animal studies. In addition, we will provide new information on the mechanisms that contribute to the development of some non-motor symptoms in Parkinson's disease, especially pain. This will allow us, and others, to explore new avenues for providing pain relief to people living with Parkinson's. Our findings will be published in scientific journals and also shared with the Parkinson's community through our contacts with the Parkinson's UK charity, for example and with the wider community through Twitter.

Who or what will benefit from these outputs, and how?

In the immediate term, our project outputs will benefit other scientists and clinicians working in the Parkinson's field by generating new knowledge to fill some of the gaps in our current understanding of the disease. By filling these gaps we hope to help in the discovery of new therapeutic avenues for treating symptoms and underlying brain pathology in Parkinson's, and thereby trigger new projects during the course of this one. Some of our strategies will involve 'repurposing' existing medicines already proven to be safe for use in humans. In these cases, these drugs could enter clinical trials within less than 5 years, potentially before this project ends. In other cases, we may uncover strategies for which no currently approved drug exists. In this way, chemists will benefit as their expertise in developing new chemical entities for this novel pipeline will be required. These new compounds will take much longer to reach clinical trials, but will certainly offer hope for the future for people living with Parkinson's disease.

How will you look to maximise the outputs of this work?

We will continue to network with fellow scientists and clinicians to exploit any findings we make to the fullest. Presentation at scientific meetings and expanding on existing collaborations, both in the UK and abroad, will ensure the impact of our outputs is maximised.

Species and numbers of animals expected to be used

- Mice: 650

- Rats: 1750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using adult rats and mice in this project. The adult stage is most appropriate as Parkinson's rarely affects individuals under 30 years of age. The brain architecture of mice and rats is well characterised and in many cases bears a close resemblance to the cellular structure and connectivity of the human brain. Their genetic makeup is also similar and they express most of the same proteins that we do. When treated with chemicals or pathological proteins, these animals also develop a condition which resembles Parkinson's. Their brain cells in relevant regions degenerate and the animals develop motor and non-motor symptoms similar to those seen in people with Parkinson's but on a much more condensed time-scale in keeping with their limited lifespan. The use of two species provides a more robust readout for drug discovery pipelines as we can be sure the drugs are not just effective in one species. In addition, they can generate different kinds of models with chemically-induced models often used in rats and pathological protein models induced more in mice.

Typically, what will be done to an animal used in your project?

Rats or mice are typically investigated initially for their baseline, control behaviour. This might involve testing the animals motor ability in simple arenas, or testing their memory for objects they see. These are non-painful, non-invasive tests that require minimal handling from the operator. After baseline scores have been gathered, animals are typically rendered mildly parkinsonian by injection of a chemical or pathological protein into a region of the brain affected in Parkinson's disease - the striatum. This happens under general anaesthesia and animals quickly recover from this surgery. The chemical injections take around 3 weeks to kill the relevant brain cells and do so by mechanisms that replicate those happening in the brain in Parkinson's disease. The pathological proteins take much longer, usually 6-9 months, to kill the relevant brain cells. However, the main protein - alpha synuclein - is the one thought to be a key trigger in Parkinson's in people so this model allows us to interrogate the pathology more fully. In both models, a partial loss of brain cells is the desired level, so the animals are only mildly parkinsonian. The animals are looked after throughout by the research team who will perform more non-painful, non-invasive behavioural tests at pre-determined intervals (usually weekly or monthly). In many cases, animals are treated with investigational drugs either while the brain cells are dying (looking here for so-called neuroprotective drugs) or after at least half of the cells have died (looking here for so-called neurorepair drugs). Drugs will be administered by injection or by mouth. At the end of the treatment period which may be a few weeks in some studies, but several months in others, the animals will be humanely killed and the brains removed for analysis to establish whether the drug treatments have worked as predicted. Other organs may also be taken if relevant to the study, such as spinal cord or bladder.
What are the expected impacts and/or adverse effects for the animals during your project?

Animals that are rendered parkinsonian do tend to lose some weight after surgery so are given additional nutrition during the post-operative week or two. This can persist for a few

weeks before returning. Immediately after surgery, animals might feel pain at the incision site. However the local and systemic administration of pain killers is routine and should keep this to a minimum. Some test drugs or the solution the test drug is dissolved in may also cause diarrhoea. If this happens, animals are given extra hydrating gels to prevent dehydration. Based upon our prior experiences, we do not expect to see any abnormal behavioural impacts in our 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 animal type)?

Because of the surgical procedure used to generate our models of Parkinson's disease the majority of our animals (~80%) will fall into the moderate severity category. However, ~20% will fall into the mild severity category. These will include animals used for working out the way a new drug is handled by the body and animals that are used to generate control tissues, for example.

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?

Parkinson's disease results from loss of cells in specific regions of the brain. This loss of cells affects the brain's ability to function properly, leading to symptoms of impaired movement and other non-motor symptoms like anxiety or cognitive deficits. The cells and multiple connections that make up the brain systems that control movement and these other relevant behaviours are complex. As such it is impossible to study this disease without the use of animals.

Cell-based models can be useful for examining the vulnerability of cells in isolation and the effectiveness of potential drugs on improving the health of these isolated cells. However, in their usual habitat of the brain, these cells are surrounded by multiple other supportive or indeed destructive cells that together control the environment and functionality of the brain. Cell-based models can offer no insight into this complex environment. They also offer no means of examining the effectiveness of new drugs to reverse symptoms relating to Parkinson's. For these reasons, we require the use animals to model Parkinson's and permit us to achieve the aims of our project.

Which non-animal alternatives did you consider for use in this project?

We have recently introduced computer-based 'bioinformatics' techniques into our research programmes, alongside the existing cell culture work and isolated brain slice work we had already incorporated. When used together these non-animals alternatives allow us to screen for anticipated changes at a single cell level and pre-select only those agents that have clear potential to progress through to the animal studies. Around a third of our activity

falls within this area. However, as noted above animals are required, ultimately to address our objectives.

Why were they not suitable?

We need fully integrated, complex brain systems that are controlling movement and other body systems affected in Parkinson's disease. Non-animal alternatives unfortunately do not offer these things, although they can be helpful early indicators of potential, as noted 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?

We have based the overall estimation on the numbers used in the past five years when funding and ongoing projects were of a similar size to those planned in this project. We have reduced these numbers by a third, compared to our previous licence, in light of the increased use of computer- and cell-based studies to inform the animal work.

For all experiments we will use a group size which is the smallest compatible with achieving statistically meaningful and robust results. These group sizes have been estimated using a combination of results from our own previous studies and those available from existing scientific literature (where available) and checked using online tools like the NC3R's Experimental Design Assistant (https://eda.nc3rs.org.uk/eda/landing), which also helps in establishing the most appropriate statistical tests to select.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have considerable experience in this type of work and have published extensively in peer-reviewed journals so are confident in the robustness of our experimental design and analysis. We have used the NC3R's Experimental Design Assistant (https://eda.nc3rs.org.uk/eda/landing) when planning the studies that were of a new design and to check that previously used designs are optimised in terms of experimental steps and analysis.

Variability will be kept to a minimum by perfecting the accuracy of our technique, using animals of the same strain and age and ensuring control animals are treated as closely as possible to those in the test group. Reduced variability facilitates use of smaller group sizes. Where fitting with our objectives, for example when examining symptomatic relief with a single treatment, we will use randomised crossover designs. These allow each treatment or dose to be tested in the same group of animals, sequentially.
Not only does this reduce variability again, it also markedly reduces the overall number of animals used.

The use of computer and cell-based screens has allowed a reduction in numbers of animals used to address our research objectives.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When undertaking studies with a completely novel design we will conduct small-scale pilot studies from which we can gain valuable insight into the minimum number of animals required to achieve our objectives. In addition, we will ensure that the maximum information possible is gathered from each animal. We will obtain multiple data readouts from each animal often comprising: measures or motor and non-motor signs; cell counts in specific brain regions; cellular and molecular markers that change with pathology or treatment. We will also use spare tissues for undergraduate and masters student projects, ensuring nothing is wasted. When reporting our data we ensure we follow the latest ARRIVE guidelines so that our findings can be confidently used to inform other researchers and therefore help longer-term to optimise the numbers of animals used in the research community (Percie du Sert N et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 2020, 18(7): e3000410).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Whilst the animal model that best recapitulates human Parkinson’s disease is the MPTP-treated primate, it is unacceptable to use this model at early preclinical stages of therapy and pathogenic investigations that form the focus of this programme of work. Hence, rodent preclinical models will be used here.

The models used most often in this project are generated by the introduction of chemicals that induce cell death in brain pathways known to be affected in Parkinson's disease. In most studies, these agents are introduced directly into relevant brain regions whilst the animals are under general anaesthesia. We induce so-called hemiparkinsonism. This involves causing pathology in one side of the brain only and produces a milder phenotype than a bilateral model would. In most of our studies we will use the chemical **6-hydroxydopamine** to cause the pathology. Moreover, we will generate a partial lesion model - aiming for around 50% cell loss in the key brain region. This degree of cell loss is sufficient for our objectives and offers a refinement from the full lesion model we often used in the past. These animals recover well from surgery. They have no impairment in their general day-to-day abilities so are able to function like healthy animals, despite having brain chemistry and cell degeneration on one side of the brain that is appropriate for these studies. They also express a wide range of mild symptoms (both motor and non-motor) that will aid in achieving our objectives. Studies in the 6-hydroxydopamine model usually last between 1 and 12 weeks, depending on the nature of the investigation; studies investigating repair of the nervous system are the longest and extend out to the 12 week

point. When treated for at least 11 days with the gold standard symptom-relieving drug levodopa, these animals develop a side-effect reflective of that seen in patients - so called levodopa-induced dyskinesia (excessive unwanted movement). This validated **model of levodopa-induced dyskinesia** will also be used in this project. Once it is induced, animals require only weekly injections of levodopa to maintain this side-effect which is expressed only for up to 2h after each levodopa injection, and not when animals are between dosing, thereby ensuring distress is minimised.

One weakness of the 6-hydroxydopamine model is its lack of clear alpha synuclein pathology. Alpha synuclein is a protein that aggregates in the brain of people with Parkinson's and is thought to play a key role in the progression of the disease. We will therefore use two additional models that are reported to exhibit this pathology. One of these introduces pathological forms of **alpha synuclein** directly into one side of the brain. The pathological protein spreads slowly throughout the brain causing mild disease state that develops very slowly. The alpha synuclein pathology appears after a month, but motor impairment and cell loss in the brain do not appear until around 6-months. While these studies are therefore much longer term, the mild phenotype is not anticipated to cause suffering or distress. Like the 6-hydroxydopamine model, this is again a partial lesion model where degeneration of 50% maximum will be achieved.

The second model displaying alpha synuclein pathology is the **rotenone** treated rat model. This model differs from the others in that rotenone can be given by injection into the body, rather than requiring surgical administration. No general anaesthesia is needed. However, as the rotenone reaches both sides of the brain eventually, this is a bilateral model and can cause a more profound motor impairment that can impact on the animal's daily activities. When this is the model of choice, animals are monitored daily to ensure they are able to move around and eat and drink without assistance.

The final class of model we may use has no pathology. These models are induced by dosing animals for a day or two with chemicals, such as **reserpine or haloperidol,** which alter the brain chemistry to prevent correct functioning of those pathways that control movement. These are mild interventions that are reversible within a few days. These models can be used for testing the ability of drugs to reverse motor symptoms but do not offer a platform for investigating the pathology of the disease or therefore drugs that can interrupt the pathology.

Why can’t you use animals that are less sentient?

We acknowledge the existence of invertebrate models of Parkinson's but while these are useful to gain information relating to the genetic influences in the disease, they have very few dopamine neurones, the ones affected in Parkinson's, and a limited behavioural repertoire so will not be useful to support our objectives. The need to monitor parkinsonian symptoms also means that the use of terminally anaesthetised animals is not appropriate. Parkinson's disease affects adults, usually those older than 30. In order to ensure appropriate translatability of our research findings, the use of immature life stages is therefore also not appropriate. Our objectives thus require adult mice and rats.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As noted above, we have refined our model by reducing the size of the lesion we induce from a full loss of cells in the relevant brain region to a partial (~50%) loss of cells. This helps to minimise the impact on the animals. We have also refined our surgical procedures

by routinely: using inhalational iso-fluorane as the preferred anaesthetic, ensuring good recovery rates and reduce mortality rates (<5%); using a micro-infusion pump to improve both accuracy and consistency of intracerebral injections; administering local and systemic pain killers prior to recovery from anaesthetic. We have also increased our monitoring frequency of animals post-operatively keeping extensive records of weight and appearance. We have incorporated more non-invasive behavioural testing of motor ability over drug-induced rotational testing to reduce stress. Animals are also exposed to their experimental environment (habituation) and apparatus plus pre-trained in any behavioural test to reduce stress during the studies. Whenever dosing, we will always use an injection needle only once, in line with best practice. Our staff are trained to the highest standards of competency and display a great deal of care towards their animals which further reduces any stress for the animals. We will continue to act upon advice from the named veterinary surgeon and other staff intimately involved with the Biological Services Unit to ensure that animal suffering is minimised. Finally, when models with potentially better clinical representation become available, we look to introduce these into our studies as refined models, as inthis case with the alpha synuclein models.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to use the NC3Rs website as our go-to- resource. This houses a valuable range of resources relating to welfare assessment, selecting and refining appropriate humane endpoints, best practice in analgesia and anaesthesia, for example. Their refinement guidelines include a range of e- learning resources and links to external publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive the NC3Rs Newsletter which has regular updates on the latest advances in all aspects including refinement. We will implement those applicable to our project after consultation with the relevant on-site animal staff.

59. Developing Treatments for myotonic dystrophy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

myotonic dystrophy, treatment

Animal types	Life stages
Mice	adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop treatments for myotonic dystrophy by 1) investigating the pathological mechanisms underlying this condition in different organs and tissues, and 2) testing novel compounds targeted to 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?

Myotonic dystrophy type 1 (DM1) is the most common form of muscular dystrophy in adults and is a highly debilitating condition, which affects 1 in 8000. There are more than 100,000 patients in developed countries, with an average life expectancy of 58 years. In addition to the health-related issues, there is a very high social cost to DM1, 50-70% of DM1 patients of working age are likely to be unemployed. Figures produced by the

Muscular Dystrophy Association USA indicate that the cost of DM to the US economy in terms of medical and non-medical expenses and lost income is roughly $450 million dollars per annum. There is currently no treatment for DM1, although some interventions are available for some complications of the disease such as diabetes and alterations in the sleep-wake cycle. However, they are limited, not effective, and do not treat the core symptom of the disease – muscle weakness and wasting. The central nervous system (brain and spine) effects of this disease have also been overlooked until recent years, despite the cognitive and psychological effects having a detrimental impact on quality of life. Being able to understand the pathological process across different organs, and how our compounds may be developed to ensure all aspects of the disease are treated is vital.

What outputs do you think you will see at the end of this project?

The ultimate aim of this research project is to develop a drug treatment for Myotonic Dystrophy. This project will provide information to support our drug development, and allow us to optimise our compounds, so after this project we can move into the final stages of drug development before testing in humans. This information will be used to support grant applications and potential collaborations with drug companies. Where not commercially sensitive, work will be published in scientific journals and presented at scientific meetings.

Who or what will benefit from these outputs, and how?

In the short/medium term the scientific community will benefit from new knowledge and improved understanding of this disease. In the mid to long-term publication of findings on the pathological mechanism of myotonic dystrophy in academic journals will disseminate this new knowledge further. In the long-term, we hope that patients will benefit from this work in the form of a treatment for a currently incurable disease.

How will you look to maximise the outputs of this work?

To advance the development of our therapeutic drugs we are working with our establishment to ensure that we have the appropriate support when we are ready to take this treatment to the next stage. In addition, we already have patents on our compounds, which may be expanded during this project. We collaborate with other groups and will publish findings in journals of good standing in the field. We will also present findings at national and international conferences.

Species and numbers of animals expected to be used

- Mice: 680

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Replicating the complex disease of myotonic dystrophy requires a mammal. Mice are the chosen species for this project as genetic models of the disease have been developed in the mouse. Adult mice are required as myotonic dystrophy is a progressive disease and time is needed for the genetic mutation to lead to the pathological processes.

Typically, what will be done to an animal used in your project?

We will use genetically altered mice that have mutations to mimic myotonic dystrophy, or in other proteins that are key for our understanding of how our therapies are working. For some of these mice, we will be able to control when the mutation is turned on – using a drug in their drinking water or food.

These genetic mice (or control mice that do not have the mutation) will typically be tested on tasks that assess movement, strength and drinking ability (requiring water restriction for a maximum of 16hrs prior to testing). And /or undergo tests of learning and memory (cognitive tests). These tests require food or water restriction to motivate behaviour (with weight loss no greater than 15% of free-feeding weight).
The motor tests are likely to be repeated daily for 5 days, cognitive tests may require additional training with sessions of approx. 30 mins in length for a number of weeks.

Following training the mouse may then be administered the compound (most likely by daily intraperitoneal injections for 5-10 days) and then testing will be repeated (as above) and tests for myotonia, which require an electrode to be inserted into a muscle will be carried out under anaesthesia, during which the animals will be humanely euthanised (terminal anaesthesia).

What are the expected impacts and/or adverse effects for the animals during your project?

The transgenic models we propose to use should not show significant overt symptoms as seen in some patients. We expect them to be able to feed, drink and groom normally. So are not expected to induce adverse effects.

There may be mild stress induced due to food, water restriction and handling for behavioural testing (movement/strength and cognitive). As well as during induction of anaesthesia for the terminal phase of the study.

There is the unlikely potential for irritation, damage, infection due to injections of compounds.

The novel compounds we are developing may induce side effects including: Disruption of gastrointestinal function (constipation / diarrhoea), Skin rash / hair loss, Mucosa inflammation, Loss of appetite / weight loss, Liver damage, Increased risk of infection. However, only small numbers of mice will be tested when a new compound is introduced to look for side effects. The mice will be monitored and if seen appropriate steps will be taken to prevent suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect the majority of animals to fall into the mild severity band (70%), however, some may reach a moderate (30%) due to potential side effects ofcompounds.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no suitable alternative to animals to model a progressive multi-system disorder like myotonic dystrophy. We need to see if our treatment strategies can improve complex behaviours, both cognitive and movement.

Which non-animal alternatives did you consider for use in this project?

We have considered and are using other non-animal alternatives where possible. This study forms part of a broader body of work in which potential drug treatments for myotonic dystrophy are tested in biochemical and cell based systems to identify those most likely to have beneficial effects. We also use computational modelling to design molecules and understand their pharmacokinetics prior to in vivo work.

Why were they not suitable?

Cell based assays and modelling cannot tell us if the compound has a beneficial effect on motor or cognitive ability in a whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Group size depends on the outcome being assessed (Approx. n=4 to 12). Wildtype littermates or wildtype background strains may be required as a control group to ascertain the normal behavioural response and non-specific effects of the compounds on these behaviours. Over the course of the project, it is likely that up to 10 large studies will be conducted assessing compounds/interventions/effects of mutation. Therefore approx. 480 mice will be used for this project. There may be additional studies required to further information about compounds dose, piloting assays or transgenic crosses = 200 mice. Totalling 680 mice for this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will carry out power calculations to ensure the optimum number of animals used to meet the scientific objectives. We have made use of the NC3Rs Experimental Design Assistant and this tool will be used to help design study protocols. Where we can we have

made sure we are using both sexes in our studies, to improve the scientific outcome and reduce the number of animals that must be bred. We have considered whether multiple objectives can be addressed with a single group of animals, and carefully considered the use of control groups. The most sensitive assessments will be used, to reduce the number of animals required. This includes the fine assessment of facial function using equipment to assess drinking, rule learning and attention using operant chambers, and the gold standard of EMG for the assessment of myotonia.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use the most efficient breeding strategy, with close monitoring of colonies to ensure we only breed animals that are required. For new compounds, transgenic lines or assays we will perform pilot studies. We will collect tissues from animals to allow a wide range of ex vivo assays to be undertaken from a single study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Transgenic models of myotonic dystrophy will be used to assess the effect of either the mutation on the mice or the effects of novel treatments. Transgenic models will also be used to understand more about the biology of the disease, and the pathways we are targeting with compounds, these may be bred with the transgenic models of myotonic dystrophy. We do not expect these models to have a significant overt effect on the health of the mice, and deficits will only be detected using sensitive tests.

Drug administration

The most refined method of administration, which suits the profile of the drug will be selected, with appropriate volumes being based on current guidelines. In most cases this will be via Intraperitoneal injection or intramuscular for compounds. It could also be orally either via gavage or in food and water. Doxycycline will need to be administered long term in inducible transgenic models, to "switch-on" the mutation.
Behavioural assessment

These are required to assess the outcomes of any genetic models or drug interventions. The most refined methods for handling will be used throughout experiments. A combination of cognitive tests will be used to assess different aspects of thinking (Maze tasks for memory, operant conditioning for things like attention and rule learning, and novel object recognition for other aspects of memory). These tests cause a minimal amount of distress for the mice. The animals can explore the equipment and voluntarily respond. Motor tests include grip strength, rotarod (which assesses endurance of the mouse to walk on a cylinder like treadmill for max. of 5 mins), assessing general levels activity and

measures of drinking function. There may be some mild distress related to handling, but this will be minimised through slowly training the animals on the tasks and giving adequate time between tests.

Food OR water restriction is necessary for some of the tasks above (operant conditioning, maze tasks, drinking function) as just giving rewards are not sufficient to encourage mice to do the tasks. However, these will be for a limited time, ensuring animals do not lose more than 15% of their body weight, and both water and food restriction will not be carried out together.

Electromyography (EMG)

In order to assess the electrical function of the muscle it is necessary to carry out EMG measures. Myotonic discharges (spontaneous runs of electrical discharges from muscles following the insertion of a needle electrode) are seen in patients and used as part of the diagnosis for myotonic dystrophy.
These abnormal discharges are also seen in the mouse models and are a key feature of the disease.

For this, a fine needle will be inserted into a muscle group (limb and/or trunk) and electrical activity recorded. This will be performed under anaesthesia, and the animals with be humanly euthanised whilst under anaesthesia, which is more refined than performing it as a recovery procedure.

Why can’t you use animals that are less sentient?

Replicating the complex physiology of myotonic dystrophy requires a mammal. Mice are the chosen species for this project as genetic models of the disease have been developed in the mouse.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Transgenic Models: We aim to use mice that do not have overt health problems, however they will be monitored and if signs of ill health / weakness are observed, weight will be monitored, wet mash will be provided to aid feeding and advice sought from the named veterinary surgeon.

Drug administration:

Experimental compounds will only be tested if they have previously been assessed for their effectiveness in cell cultures. The maximum dose will be informed by the pharmacokinetics of the compounds, and will be tested on limited numbers of animals (1-2) initially to ensure they do not cause adverse effects. Administration routes will be dependent on the bioavailability and half-life of the compounds. As these are initial studies on these novel compounds bioavailability needs to be ensured, so it is likely that intraperitoneal or intramuscular injections will be used. Maximum volumes will follow current guidelines and the duration and frequency of treatment will be limited considering the overall impact on the animal (see protocols for exact limits). The compound will be of appropriate pH (4.5 – 8) and free from pathogenic material. During the period of compound administration body weight will be assessed, as well as daily monitoring of general condition. Swift and appropriate action will be taken if adverse events are seen, for example discontinuing drug administration and humane killing of the animal

Doxycycline was selected as it has fewer side effects than tetracycline, and it will be administered via the drinking water, as this is more refined than repeated injections. Water consumption will be monitored to ensure that it is not reducing water intake.

Behavioural assessment.

Habituation and handling: The most refined methods for handling will be used throughout experiments, and animals will be habituated to handling prior to experiments to reduce stress.

Each **motor assessment** can be assessed a limited number of times, with a defined rest period, to prevent exhaustion (see protocol section for limits), and animals will be pre-screened to ensure they are capable to perform the taskss.

Drinking assessment (lickometery): If animals are housed in these cages for extended periods (>2h) a shelter or tube will be provided and the temperature of the room will be increased or warming mats will be placed under part of the cage to prevent hypothermia. As bedding can not be put in these cages as it prevents the apparatus from recording accurately.

Food and water restriction:

Mice will not undergo both food and water restriction simultaneously. We will ensure mice will have bedding to help thermoregulation during periods of food and water restriction.

Food restriction represents the most refined method of motivation as hunger causes the least amount of distress. The animals will be without food for a minimum of 4 hours and up to a maximum of 23 hours per day for a maximum of 12 consecutive weeks. We aim to refine this by employing the minimal amount of restriction time required to motivate the animals to perform in the behavioural test. Weight loss will not exceed 15% of free-feeding body weight and animals will be returned to free-feeding status if weight can’t be maintained at appropriate levels.

Water restriction represents the next most refined method to motivate performance, and can be required to assess drinking function. For other tasks it is preferred in situations where food restriction is not motivating performance effectively, as it can achieve stable changes in body weight more effectively.
The animals will be without water for a maximum of 16 hours prior to testing for a maximum of 5 consecutive days. We aim to refine this by employing the minimal amount of restriction time required to motivate the animals to perform in the behavioural test. Weight loss will not exceed 15% of free-feeding body weight and animals will be returned to free water status if weight can’t be maintained at appropriate levels. Both restriction regimes have been published extensively, and there is evidence that water restriction results in better performance in operant conditioning tasks.

Electromyography (EMG)

This will be carried out under terminal anaesthesia, with careful monitoring of anaesthetic depth. This is more refined than performing a recovery procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Good Laboratory Practice and relevant LASA and NC3Rs guidelines to ensure experiments are conducted in the most refined way. Work will be reported in line with the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am on the 3Rs working group within my institute, so am aware of advancements across the sector. We regularly monitor the 3Rs webpage, and receive email updates. We will implement any changes we learn of that could be of benefit to our animals.

60. Sensory and emotional processing of pain in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pain, Nociception, Anxiety, Depression, Pharmacology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to achieve a deeper understanding of the sensory and emotional component of pain states. This project aims to investigate the neurobiology of pain, identify new molecular targets and introduce novel approaches to treat chronic pain to offer better pain relief and improve overall quality of life of people living with pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Pain is a protective mechanism necessary for survival that informs us of tissue damage that will require time to heal. While normally of biological benefit, pain can sometimes persist, either after healing or due to on-going disease, and become chronic; such conditions are particularly difficult to manage with current therapeutic approaches. Pain

also triggers aversive and threatening psychological feelings and patients in pain are more likely to become depressed and anxious, have disrupted sleep patterns and generally have a poor quality of life. It has been estimated that about 365 million days are lost per annum in the UK alone through pain related illness.

Pain commonly presents as a result of an injury or a disease; however, it is also a separate condition in its own right, not just an accompanying symptom, affecting millions of people worldwide (Vos et al, 2017). In Europe, chronic pain affects at least 19% of the population, reduces the quality of life and the ability to work, therefore presenting a huge social and economic burden (Breivik et al, 2016). The major types of clinical pains arise from surgery, trauma and disease and can be classified as pain from inflammation (rheumatism and arthritis), nerve injury (chemotherapy-induced pain, post-herpetic neuralgia, diabetic neuropathy, AIDS), and cancer. It has been reported that pain is poorly controlled in up to 4.2 million patients dying with cancer. One of the reason for this is the clinical fear of producing an addicted state if opiates have been prescribed. Among patients who suffer pain, 33% had pain all or most of the time and 87% of those with pain rated it as moderate to severe. Pain control is still in its infancy. Only 33% of patients with nerve injury achieve pain relief with current pharmacological approaches and this relief is often only partial and accompanied by side effects. 20% of people with cancer have movement-related or neuropathic pain, and perhaps one-third of visits to Pain Clinics are from patients with similar pain not due to cancer.

Our goal is to achieve a deeper understanding of the neurobiology of pain states in order to offer better pain relief and improve the quality of life of people living with chronic pain.

What outputs do you think you will see at the end of this project?

It is usual to associate pain with injury and to assume that once the injury has healed the pain will dissipate. In fact, 19% of Europeans suffer from moderate to severe pain that does not resolve even though the injury has healed. Injury, whether though surgery or accident is known to alter pain sensitivity but how this occurs is not known. In the short-term our work aims to uncover the mechanisms responsible for this altered pain sensitivity and therefore is likely to identify potential biomarkers for the susceptibility to chronic pain in vulnerable individuals. By combining different behavioural paradigms and molecular biology in mouse models of pain, we will demonstrate that manipulation of physiological pathway, e.g. autophagy, can alleviate the pain and comorbid changes observed in patients. Moreover, research into the control of pain states has identified neuronal pathway connecting the spinal cord and brain that are key to the regulation of on-going pain. It was shown previously that destroying these pathways alleviated on-going persistent pain in rats and companion dogs. This approach has however not translated to human patients. At the end of this work we will demonstrate that our approach, using constructs made from botulinum toxin can silence but not kill pain-signalling neurons.

Pain has two main components, a sensory and an affective or ‘emotional’ component and both have to be considered when searching for new analgesics. Using the behavioural paradigms described in this application, we will fully characterise the two component of pain and identify novel and effecting analgesics for the treatment of pain.

New treatments for chronic pain in humans will emerge from our studies that will use various approaches to obtain a better understanding of the neurobiology of pain state and uncover new targets and new approaches for pain therapies. We will identify new pathways to analgesia and identify new drugs for the control of persistent pain states that are more effective that the currently available analgesics.

While clinical trials are currently testing whether the modulation of autophagy, a major intracytoplasmic protein degradation pathway, could improve the treatment of cancer and neurodegenerative diseases, targeting autophagy to reduce chronic pain has been poorly investigated. At the end of this work, we will demonstrate that manipulation of autophagy improves not only the ongoing pain but also the emotional state of injured mice. This will be fully investigated before moving towards clinical to meet clinical needs.

We have recently demonstrated that botulinum conjugated to dermorphin is as effective as morphine but without the side effects of tolerance and addiction in mice. We will now explore the reversibility and toxicity of this treatment before moving towards translation. For this reason, when the effect of botulinum-based construct will wear off, analgesia will be re-instated with a second injection of the constructs.

We believe that, in the long-term, new treatments for chronic pain will emerge from the different approaches outlined in this application and we will identify clinically relevant approaches opening the way to future translational research.

The outcomes of our research will be published in open access journals and presented at national and international congresses attended by basic scientists and clinicians. Our work will undoubtedly provide benefits to a number of people.

Who or what will benefit from these outputs, and how?

Our group first of all will use the data produced with this project to obtain further funding for our research.

Other researchers in the field will also directly benefit from the advancement of the understanding of the neurobiology of pain states that our work will provide.

Pharmaceutical companies and NIH have already shown their interest of our work and are constantly on the lookout for new potential targets for the treatment of persistent pain states. Our work is of substantial translational value and of particular importance in the current battle against addiction and drug overdose that has been triggered by over-prescription of opioids for chronic pain in USA.

The longer term aim is for clinicians and patients to benefit from our work, in particular in clinical settings where patients are constantly looking to understand their condition and questioning how proposed treatments work. Chronic pain patients will also directly benefit from the identification of novel targets when new drugs are available for clinical use.

How will you look to maximise the outputs of this work?

The outcomes of our research will be published in open access journals and presented at national and international congresses attended by basic scientists and clinicians.

We will use the data produced with this project to obtain further funding for our research. Moreover, pharmaceutical companies and NIH have already shown their interest of our work and are constantly on the lookout for new potential targets for the treatment of persistent pain states. Our work is of substantial translational values and of particular importance in the current battle against addiction and drug overdose triggered by over-prescription of opioids for chronic pain.

Species and numbers of animals expected to be used

- Mice: 2550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All experiments will be carried out in rodents in order to compare the data with previous and ongoing studies from our and other laboratories. The anatomy, neurochemistry and physiology of pain control systems are well documented and there are well-established models of chronic pain in this specie. As such, the use of rodents will facilitate the interpretation of the data in a meaningful way. Moreover, with the wide range of existing transgenic, mutant and knockout models make mice an irreplaceable model for our studies.

Pups will be used only when injection of an inflammatory agent is required to prime the nociceptive system at this age.

Typically, what will be done to an animal used in your project?

To address our 3 Objectives, we have designed our project around classical and translational models of pain (e.g. models of disease in which pain is a symptom). We also use a range of behavioural approaches to monitor animals' well-being and the development of the hypersensitive states. Anumber of interventions are required before or following the induction of the pain state to reduce animal pain behaviour and/or to allow us to identify the signalling pathways involved in the pain behaviour.

We use the least severe pain model possible. Acute Pain measurements generally requires injections of substances such as capsaicin (the pungent 'hot' component of chili peppers) or formalin without anaesthetic so that behavioural assessment can be made of acute pain sensitivity within minutes after injection. Inflammatory pain can be modelled with paw injection of substances either for short acting inflammatory lesion or for a longer lasting inflammatory state where maintenance of the condition is being studies. Neuropathic models are more severe models, although the increased sensitivity is relatively modest albeit long lasting. We also use a model with strong translational value such as chemotherapy. In some cases, the use of sham operated animals will be required as a negative control. The sham surgery will be used to control for pain from superficial structures. Mice undergoing sham surgery, compared to naïve animals, will experience nociceptive signalling from superficial structures but not from deep tissue.
For a particular experiment, we chose the model most likely to help us answer our scientific question. When exploring early molecular pathways activated in the early stages of a pain state, which is a potential key step in the identification of novel targets for the treatment of pain states, we often use acute models. Nociceptive stimulation results in molecular changes in the cell body of the primary afferents, in the dorsal horn of the spinal cord and in brain areas relevant to pain signalling and it is then possible to map changes in signalling pathways. However, when we are interested in the mechanisms that maintain chronic pain states in humans and the role of higher brain structures in the maintenance of these pain states, we use classical models of persistent pain, both of inflammatory and

neuropathic nature, well-described in the literature. In this instance, it is important to monitor animal behaviour for up to 6 months after the induction of the pain state, a time at which others have demonstrated the development of comorbidities such as depression and anxiety in rodents with persistent pain, a particular focus of this licence.

Behavioural assessment will occur before and after the induction of pain state. The development of the hypersensitivity will be investigated with behavioural methods that are non-invasive and cause very little distress. Behavioural test can start up to 2 weeks prior the induction of the pain state or prior to any intervention that might precede the induction of the pain state.

A number of intervention will be used before and/or following the induction of the pain states to reduce animal pain behaviour and/or to allow us to identify the signalling pathways involved in the behaviour.

At the end of our experiments, we always take tissue for molecular analysis and for confocal microscopy analysis. We will use great care in ensuring that mice are well maintained and suffer minimal distress as well as using best practice surgical procedures. Environmental enrichment, such as carton tubes and wooden artefacts, will be provided though this may be tailored so as not to affect experimental measurements. Such environmental enrichment stimulates the animals and contributes to their physical and psychological well-being. At each stage anaesthesia will be considered as described more fully in protocols below.

What are the expected impacts and/or adverse effects for the animals during your project?

We have chosen models that have been widely used in the pain field for many years. This will allow us to build on previous data and to compare our work with that of others and will ensure that we do not need to characterise novel models that might not be suited for the study of both pain mechanisms and translational approaches for the treatment of chronic pain in humans.

We will use pain models that generally cause some mechanical hypersensitivity and threshold testing procedures will be used to monitor for obvious signs of distress. Injection of substances such us formalin or capsaicin induces a pain state that will resolve within one week. Chronic stimulation with eg CFA, carrageenan or cytokines produces a maintained mechanical and thermal hyperalgesia. The inflamed area swells and becomes sensitive and shows signs of erythema. The area of inflammation will be regularly inspected but in our experience has never developed into an open wound. Injury to peripheral nerves leaves some motor impairment and local soreness at the site of incision.

Animals will be monitored closely for 2-4h following injury and the observed daily following the induction of the pain state.

Our models are moderate in that they generally produce a moderate increase in mechanical sensitivity and we will not use the more invasive models unless absolutely necessary to replicate an experiment in the literature. We will use local anaesthetic and analgesic whenever possible but only when this will not compromise the experiment. At the termination of the experiment animals will be killed (to take fresh tissues for molecular analysis) or perfused, when microscopy is going to be used to assess changes in the nervous system.

Animals will be humanely killed if they exhibit any of the following signs for more than 24h following appropriate interventions and the NVS and/or NACWO will be consulted: hunched appearance, reduced activity, altered gait, piloerection; or continued weight loss of (greater than 10%) despite supplemental feeding.

Once animals lose 5% weight, loss monitoring will be increased and steps taken to prevent further weight loss and/or enable weight to be regained. Wet mash will be added directly into the cage.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect 100% of animals on this project to develop 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?

In most of our studies it is essential to use animals rather than cultured cells in dishes. The use of animals (male and female rodents) is essential for this application because we are working on a complex experience, pain, which is processed at various levels in the central nervous system and it cannot be modelled in vitro. Rodents are known to display long-term emotional-changes in long-term pain states similar to that seen in humans. Therefore, rodents are an excellent model to study the overall long-term behavioural and molecular changes associated with the developments and maintenance of persistent pain states. Moreover, they are also an important and crucial step to investigate the actions of novel treatments for pain states.

Which non-animal alternatives did you consider for use in this project?
Although non-protected invertebrate species such as *Caenorhabditis elegans* or *drosophila melanogaster* do contain neurons, they do not display appropriate behaviours, nor do their nervous systems contain the appropriate structures or complexity, to be a useful model of pain in humans.

Why were they not suitable?

In most of our studies it is essential to use animals rather than cultured cells in dishes. The complex diseases we study require that we look at the behaviour of the whole animal.

All our experiments will be carried out using rodents as these animals also enable the use of the advanced genetic tools needed to dissect the cellular circuitry of the nervous system in a precise, neuron subtype-specific manner.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experts in experimental design and always use both our experience and statistical approaches to estimate the number of animals we need in our experiments not to waste or overuse animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We routinely use randomisation and blinding in our experimental designs to prevent subjective bias. The number of animals used during the course of this project will be minimised by attention to experimental design and statistical power calculation using previously determined measures of variance and effect size estimates based on previous and pilot experiments. *n* numbers will be calculated using online resources including the NC3Rs experimental design assistant.

Often we use both sexes of animal in our experiments, so our results can be applicable to the whole population. This is also taken into account when estimating our sample size. We also seek specialist statistical advice where required for new study designs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our experience in the field and the appropriate use of pilot studies always ensure that we keep numbers of animals to a minimum but that we use appropriate sample size. Moreover, when possible and if a number of experiments are run at the same time, controls are shared across study groups to reduce further the number of animals used. Novel test compounds provided by our collaborators are never used in animals without being first tested in vitro and in a small group of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents are an appropriate species for studying the functional of neural circuits. Rodents are mammals and sufficiently similar to humans to be of relevance for human biology; secondly, they have been used extensively as model organisms so this study can build upon earlier experimental results.

We use models of pain or altered sensation that are well validated and clinically relevant. In our models, the severity limit is never greater than ‘moderate’ and attention will be given to minimising the irritation and pain felt by the animal where possible. We will use pain models that generally cause some mechanical hypersensitivity and threshold testing procedures will be used to monitor for obvious signs of distress. Experiments will not be continued for longer than is absolutely necessary. Models will be selected on the basis of their relevance to the problem being studies and attention will be paid to reducing suffering to the lowest possible level. We will not use more invasive models unless is absolutely necessary to demonstrate the analgesic properties of a specific compound in specific model of pain.

Our pre-emptive approach (Protocol 6) to silence key neurons in the nociceptive pathway will avoid unnecessary suffering in the animals. Moreover, local anaesthetic may be used at sites of incision as an analgesic and systemic analgesics will be given routinely unless there is evidence that they would interfere with the development of the model or our experimental outcomes.

We have chosen models that have been widely used in the pain field for many years. This will allow us to build on previous data and to compare our work with that of others and will ensure that we do not need to characterise novel models that might not be suited for the study of both pain mechanisms and translational approaches for the treatment of chronic pain in humans. Importantly, we have started engaging with clinicians and with the patient community through patient and public involvement activities to ensure that our chosen models have translational value (i.e. that the animals display patient relevant symptoms),as we want our work to be of maximal benefit for the patients.Our behavioural approaches will also provide us with valuable information with high translational value.

All experiments will be performed on mice, as the ‘simplest’ mammalian model available, however if a larger size and/or increased intelligence of rats will be required to make it more likely that an experiment is successful, then we will submit a project licence amendment to add rat as necessary.

Why can’t you use animals that are less sentient?

Whereas nociception is a well-known process in all vertebrates, animal models with lower sentience (e.g. fish) cannot be used here. It has been demonstrated that fish lack the neocortex known for processing sensory information in mammals and therefore they cannot be used to achieve the aims of this project. Moreover, it is still not clear how pain is perceived and how it affects fish behaviour.

Mice are mammals and sufficiently similar to humans to be of relevance for human biology; secondly, they have been used extensively as model organisms so this study can build upon earlier experimental results. The anatomy, neurochemistry and physiology of pain control systems are well documented and there are well-established models of chronic pain in these species. As such, the use of rodents will facilitate the interpretation of the data in a meaningful way.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use great care in ensuring that mice are well maintained and suffer minimal distress as well as using best practice surgical procedures. Environmental enrichment, such as carton tubes and wooden artefacts, will be provided though this may be tailored so as not to affect experimental measurements. Such environmental enrichment stimulates the animals and contributes to their physical and psychological well-being.

We will use local anaesthetic and analgesic whenever possible but only when this will not compromise the experiment. The use of anaesthesia for ip injections of chemotherapeutic substances will reduce stress in the animals and improve animal welfare.

At the termination of the experiment animals will be killed by a schedule 1 procedure (to take fresh tissues for molecular analysis) or perfusion, when microscopy is going to be used to assess changes in the nervous system.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will regularly monitor the Home Office web page for technical advice and guidance related animal research.

All the interventions listed in this application will follow the N3CRs guidelines. LASA guidelines on administration of substances will be used.
LASA 2017 Guiding Principles on Record Keeping for Personal Licence Holders will be used to achieve a good project management

The ARRIVE Guidelines will be used to improve the quality and reliability of our published research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Home Office website (https://www.gov.uk/guidance/research-and-testing-using-animals) will be visited regularly to stay informed about Home Office circulars, Statistics (annual statistics and trends), Publications, Guidance documents and Codes of Practice.

Moreover, regular meetings with Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO) will be hold.

61. Immunity to viruses and virus-based vaccines

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

virus, immune system, inflammation, vaccination, cancer

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo, aged
Hen eggs	embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate how manipulation of the immune response to viruses can be beneficial in two different but related scenarios, namely: 1) if the immune response to a virus causes disease to the host and 2) if a virus is a major component of a vaccine that is designed to protect against cancer or a different (heterologous) viral infection and associated 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 COVID-19 pandemic has demonstrated how harmful immune responses induced by viral infections can have deadly consequences. Understanding how to dampen these responses can lead to the development of new therapeutics to treat virus-infected individuals. The COVID-19 pandemic has also shown how important vaccines that are based on viruses are - both the Oxford/AstraZeneca and Johnson & Johnson COVID19 vaccines are (safe) viruses. Viruses have great potential as vaccines not only for infectious diseases but also cancer. Understanding how to induce the best possible immune

response against these 'viral vaccines' will maximise the chances of future success of these preventative medicines.

What outputs do you think you will see at the end of this project?

The proposed studies will advance our understanding of how viruses induce immune responses, how these responses can cause damage if they are not regulated properly, and how virus-induced immune responses can be harnessed in vaccination strategies. These studies of both virus-induced inflammatory responses and virus-based vaccines will undoubtedly lead to numerous publications, as evidenced by our track-record as a laboratory. We have a track-record of publishing data from animal studies even if the findings show no effect, ensuring that other scientists do not perform unnecessary experiments on animals.

During this licence we will work with the University innovation team to develop patents based on our findings, and will work with pharmaceutical partners, sharing our data where our results suggest that drugs already exist that could be used to treat harmful immune responses triggered by viruses. Our studies will also inform the development of vaccines that may be generated for future translation into human studies at the end of this project.

Who or what will benefit from these outputs, and how?

Studies of Virus-Induced Inflammation: These studies will inform the development of anti-inflammatory strategies in the treatment of virus-infected individuals. Depending on what we find, it is conceivable that certain drugs may already be used to treat individuals in inflammatory diseases such as arthritis. Therefore, it is possible that research in this 5-year project may provide evidence to support clinical trials at the end of this project that examine the use of existing drugs in virus-infected individuals. It may also discover new pathways to target where existing drugs are not available. These approaches could lead to patient benefit in ~10 years.

Virus-Based Vaccines: Vaccines that are based on viruses such as adenovirus and cytomegalovirus represent exciting approaches to induce potent immune responses that could be directed to other viruses such as influenza, but also cancer. With colleagues at the home establishment, we are developing different virus-based cancer vaccines and testing them in parallel in human systems.
Clinical colleagues are already performing clinical trials using virus-based cancer vaccines. Therefore a clear pipeline exists that means that performing clinical trials using virus-based vaccines at the end of the 5-year project is a possibility. Studies looking at the combination of vaccines and other methods of immune manipulation, or using more novel vaccines (e.g. those based on cytomegalovirus) may take longer (6-10 years)

How will you look to maximise the outputs of this work?

In all cases we will disseminate our findings through publication in high-profile manuscripts and also using open access preprint vehicles such as Biorxiv, thus enabling access for all to our research free- of-charge. As stated above, we will also continue to publish negative/unsuccessful results. For studies of virus-induced inflammatory responses, as stated above, we will collaborate with pharmaceutical companies to identify druggable pathways that could be used to treat harmful immune responses triggered by viruses. We have clinical collaborations established to enable effective translation of our findings regarding virus-based cancer vaccines into patients. We will also disseminate our findings to the general population through press releases and interviews. We will also patent all

relevant findings to aid the development of products as a consequence of research outlined in this project.

Species and numbers of animals expected to be used

- Mice: 9200
- Hen Eggs: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the lowest vertebrate group in which the viruses and virus-based vaccines that we will use have been well characterised. Numerous reagents exist to therapeutically target molecules of the immune system in mice and modified mice lacking genes that may influence virus-induced immune responses exist. Furthermore a large number of conditional knockout mice also exist which will enable investigation of specific regulatory pathways in cell types that are essential in the regulation of antiviral immune responses. In most of our studies of both virus-induced inflammation and virus-based vaccines, we will use adult mice as their immune systems have matured and are best characterised. However, we also wish to study neurological inflammation that is induced by cytomegalovirus. In humans, cytomegalovirus is the most common congenital infection, causing devastating life-long neurological defects. In mice, a well-characterised model exists for congenital cytomegalovirus infection that involves infecting newborn mice with mouse cytomegalovirus (MCMV). Thus in some experiments, we will infect newborn mice with MCMV and study virus-induced inflammation.

Typically, what will be done to an animal used in your project?

In the case of studies of virus-induced inflammation, a mouse will be infected with virus either by either injection or administration in the nose. Signs of illness will be monitored and mice may also be treated with a substance to manipulate the immune response to reduce inflammation. These substances will be administered by injection, or by intranasal administration or through drinking water. In some experiments, the long-term behaviour of the mice will be measured after ~9-12 months, studying, for example, how an infection can affect mouse cognition.

For vaccination studies, a typical experiment would involve vaccinating a mouse with a virus-based cancer vaccine, usually by injection, and then bleeding mice monthly to measure the induction of the immune response. After several (up to 9) months, the mice would then be challenged either with an infection such as influenza, or a cancer (either a cell line or using mice that you can induce cancer development in). Development of the cancer or infection will be assessed over time (typically days for infections, weeks for cancers) before mice will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

For virus-induced inflammation, mice will experience mild to moderate weight loss in the first 7-10 days of infection. If virus infections are performed in mouse strains that are susceptible to Alzheimer's disease, some of these mice may show accelerated cognitive decline, as observed through readouts such as altered novel object recognition. In the case of neonatal cytomegalovirus infection, mice may lose weight but will likely demonstrate abnormal brain development. Here, most experiments will only last for 2-3 weeks before the mice are humanely killed. Therefore mice will not experience long-term neurological abnormalities.

For vaccination studies, mild weight loss may be observed after vaccination for the first 3-5 days before resolution although many vaccinated mice will experience no signs of vaccination/infection. During challenge with viruses, weight loss may be observed as with virus-induced inflammation studies. For cancer vaccine studies, tumour growth will likely be observed in mice. These will be measured for up to 4 weeks post-cancer challenge. Some mice in cancer vaccine studies may experience no tumour development however.

Expected severity categories and the 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 studies of virus-induced inflammation, based on previous experience with these models, we expect ~40-50% of mice to experience moderate weight loss as a consequence of virus infection. In vaccination studies where we are challenging with infectious agents, we expect 20-30% to experience moderate symptoms during infectious challenge. In the case of cancer vaccines, we expect 60% to experience moderate severity as a consequence of tumour induction.

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?

Immune responses induced by infections are very complex and there are no *in vitro* techniques that recreate an intact immune system. Because these studies cannot be replaced by using either computer modelling or *in vitro* experiments involving cell lines or animal or human tissue, live animals must be used. This proposal will investigate complex inflammatory networks that are triggered in response to virus infections. It will also study how viruses can be used as vaccines to protect from other viruses and from cancer. These networks will likely involve numerous cytokines and pathways that act on a multitude of immune cell types in different organs of the body.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we will perform *in vitro* infections using systems recently developed in our laboratory. Specifically, during the last PPL we developed human genetic systems to study virus- induced inflammation that is based on human stem cell technology. We validated this model by showing that modelling an influenza-induced inflammatory response in this human system showed major parallels to inflammatory pathways triggered during influenza infection in mice. Therefore, we will use these in vitro models wherever possible, particularly for studying inflammatory immune responses to inform decisions of what experiments will be worthwhile performing in mice.

We have also, as a laboratory, moved away where possible from the use of *in vivo* generated mouse CMV (MCMV) stocks. Nearly all of our studies (both inflammation and vaccine work) now utilise *in vitro* generated MCMV rather than virus generated in mice.

During the lifetime of this project I hope to further reduce in vivo work, particularly the neonatal CMV infection work where we examine the impact of virus infection and associated neurological inflammation on neonatal brain development. In particular, we have been awarded a seedcorn funding to establish a human brain organoid-based HCMV infection system to study CMV-induced inflammation in the brain. If validated, this will form the foundation of an additional in vitro assay in our pipeline of studying CMV-induced inflammatory pathways where, if a given anti-inflammatory intervention strategy fails to affect inflammation within these brain organoids, they will not be tested in vivo. We hope that this model system will also support more in vitro mechanistic studies of how CMV induces inflammation in the brain.

Why were they not suitable?

To assess the true complexity of inflammatory immune responses and, critically, to be able to assess the therapeutic efficacy of approaches to target virus-induced inflammation, small animal models are required. Furthermore, for testing virus-based vaccines and for understanding whether these can protect from cancer, we need to test these approaches in mouse models of cancer. Cell-based systems such as those from human stem cell approaches cannot recreate the complexity of an immune response induced by a vaccine and then be used to assess whether the vaccine-induced immune response can control cancer development. This is far too complex to model *in vitro*. Moreover, 3D-based systems or computer modelling also could not be used to recreate the full complexity of an immune response induced by viruses.

For some of our studies, we still need to use MCMV stocks that are isolated from the salivary glands of mice. This is because salivary gland virus best infects the salivary glands itself. This is important because shedding of HCMV in human saliva is a main source of virus that infects unborn babies and causes life-long neurological defects in these individuals. We want to identify how we can stop this process and therefore need to study CMV infection in the salivary glands using the optimal mouse models available, hence some of our work will still require virus that we have grown in the salivary glands of live 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?

Experimental designs to be utilised are simple and have been used, with slight variations, in our laboratory for a number of years. Indeed, I personally have been working with the influenza and CMV mouse models for 20 and 15 years, respectively. Therefore, for most experiments little optimisation of regimes is required, keeping the number of animals required as low as possible. However, when we develop new approaches (such as testing a new type of virus-based vaccines) we routinely consult the departmental statistician to ensure that our experiments for the particular approach are powered correctly.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All PhD students and post-docs in the lab will have performed a course on use of statistics in biology and will be well versed in use of "power calculations". This means that they will be able to use "power calculations" to work out what the minimum number of animals is needed in order for a given experiments to produce a meaningful (statistically significant) result. This is important, not just for making sure that no more animals than necessary are used, but also because using too few mice means that it might not be possible to draw a conclusion from the experiment. As stated above, when we perform new approaches in our lab, our lab members also consult with our institute statistician to obtain external expert statistical advice.

Members of the lab also use a set of guidelines called "ARRIVE" guidelines found on https://arriveguidelines.org/. These are an extremely useful set of recommendations which are designed to help researchers ensure that they perform their animal work in the most robust way possible. The purpose is to enhance the quality and reliability of the findings of individual labs in order that their work can be more easily reproduced by others. These principles form the cornerstone of animal work performed in our lab. We will also use the NC3Rs Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

There are several measures we will take to reduce animal usage:

We will share control groups wherever possible. For example, we aim to test multiple anti-inflammatory strategies concurrently with the same single control group

We will perform longitudinal analysis on the same mice. This is particularly relevant for vaccine studies where we study vaccine-induced immune responses over time by taking regular blood samples and measuring vaccine induced immune responses

As described in detail elsewhere, for studies of viral and cancer vaccines, we will study both vaccines at the same time and use the other group as control for much of our analysis. For example, when measuring induction of cancer-specific immune responses we will use our flu vaccine group as the cancer-irrelevant control. When measuring influenza-specific immune responses induced by our flu vaccine, we will use our cancer vaccine as our influenza-irrelevant control. Thus, we will concurrently develop two different vaccine approaches at the same time using the same mice.

Randomisation is also used to ensure validity of results. Due to the nature of the tumour models that are used, whereby tumours arise at different times in individual animals, experiments are often performed in sub-groups (enabling randomisation within blocks). This approach builds additional robustness to the experimental findings as any conclusions drawn come from findings which have withstood the natural variation occurring within experimental units.

Variation amongst mice / experiments is kept to a minimum by conducting procedures at a similar time of day, using uniform and well validated techniques and equipment. In all cases individual mouse variation e.g. body weight, tumour size etc, are taken into account.

Where possible, experiments are performed in a blinded fashion. Animal samples taken to the lab will be coded and key measurements conducted by a researcher who is blinded to the experimental code e.g. measurements of immune cells in tumours or virus-induced weight loss. Tumour volumes are measured by technicians who are not aware of what (or whether) mice received interventions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 predominantly use the mouse cytomegalovirus (MCMV) and influenza models as these are the best-characterized small animal models for these infections. Both infection models induce comparable inflammatory responses to those observed in humans. Importantly, the virus strains used in animal models for both MCMV and influenza are well characterised, so we know exactly how much virus to give to mice to induce a certain amount of disease that is well within UK Home Office restrictions. Also, using mice enables us to administer treatment strategies for virus-induced inflammation with minimal distress such as simple subcutaneous and intraperitoneal injections that we typically use that do not require anaesthesia. Similarly, for virus-based vaccine work, the mouse enables relatively painless sub-cutaneous injections during vaccination. Here, mouse models of cancer are also well characterised which will enable the examination of virus-based cancer vaccines with minimum suffering to animals.

In all procedures animal suffering will be minimised through good animal handling techniques and strict adherence to monitoring procedures. These monitoring procedures ensure that adverse effects associated with viral infection and vaccinations will be minimized, this keeping pain and distress caused by infections to an individual animal at a minimum.

Why can’t you use animals that are less sentient?

For many experiments we will require an adult immune system as this will model best the patients that often suffer from severe viral diseases such as during influenza infection. The same is true for our vaccination studies where our aim is to generate vaccines to target cancer and respiratory diseases where older individuals are more likely to suffer from these diseases and thus will be the primary target population for these therapeutics. Importantly, however, for CMV infection studies, we will be increasingly using the neonatal mouse model for some of these studies, thus using more immature, fewer sentient mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All new techniques (or combination of techniques and/or immune intervention strategies) are subjected to scrutiny with respect to harms to the individual animal. Score sheets are updated to reflect any relevant observations relating to animal welfare. We also work closely with the animal technicians and NACWO to ensure that all parties are aware of potential adverse effects and how to respond accordingly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidelines for the welfare and use of animals in cancer research, Workman et al, British Journal of Cancer, 2010, 102(11):1555-77

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay informed about advances in the 3Rs (reduction, refinement and replacement) through published literature, such as the recent development of brain organoids as a model for CMV-induced brain inflammation. If we validate this as a model for studying brain inflammation induced by CMV, we will likely replace significant amounts of neonatal MCMV work.

Students and post-docs in the lab also attend and participate in workshops which are run by the "National Centre for Replacement, Reduction and Refinement of Animals in Research" - this provides the lab with the opportunity to learn about the latest innovations which help reduce the need for animal models or which improve experimental designs such that fewer animals are needed.

62. Unravelling the role of tumour microenvironment in mediating resistance to anti-cancer therapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Tumor Microenvironment, Therapy, Extracellular vesicles, Metastasis

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's the aim of this project?

To better understand how tumours respond to therapies and why current therapies have limited efficacy in some cases, often associated with cancer spread (metastasis). Importantly, this knowledge will provide the rationale for the design of new, improved combinatorial therapies that we can take to the clinic and improve the management of this deadly disease (i.e. better response rates, improved survival of cancer patients).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

While we have made significant progress in treating some types of cancer successfully, cancer remains one of the most life threatening diseases humans encounter. Over the last two decades we have discovered that cancers that develop in a particular organ eg breast, represent multiple different types of cancer, each of which develops slightly differently and needs different therapeutic strategies. Notably, it is now widely accepted that tumours are not just "bags" full of cancer cells, but contain several other non-malignant cells such as immune cells and endothelial cells that form blood vessels which supply the tumours with nutrients, thus supporting their growth. Many anti-cancer treatments show limited therapeutic efficacy due to alterations (either pre-existing or acquired during therapy) on these normal cells of the tumour, which consist the tumour microenvironment. Tumours that are refractory or resistant to therapy have higher chances of spreading to secondary sites, leading to metastasis. Thus, cancer remains an incredibly complex disease and understanding why some tumours do not respond to current therapies will help us identify better therapeutic strategies that will eventually stop cancer from being a life-threatening disease.

What outputs do you think you will see at the end of this project?

We expect multiple beneficial outputs from our programme of work:

We will generate significant amounts of new biology that we will share with the scientific community;

We will generate multiple publications that we will ensure are available on open access so that as many people as possible can read our research, learn from our experiences and data, and use the data to generate new hypotheses; It is likely that our programme will generate/characterise one or more new compounds/drugs/tracers that will be translated into humans in clinical studies and trials;

Our research will allow us to attract more grant income that will enable us to train more new young scientists in the field of in vivo research.

Who or what will benefit from these outputs, and how?

Our programme of work will benefit many stakeholders:

The scientific community will benefit from our publication of and public speaking of our data;

The public will benefit from the revelation of our new biology and also our development of new therapeutics. By enhancing knowledge and providing new therapeutics clinical management of patients will change for the better;

A new cadre of scientists trained in the development of, management of and conducting animal experiments will be generated as a consequence of this programme;

As our data will inform scientists in our fields of the new biology, certain animal experiments that would otherwise have been conducted will no longer be necessary reducing the numbers of animals used.

How will you look to maximise the outputs of this work?

The knowledge and experience, good and bad, that we develop within the programme will be made available as widely as possible. This will include research manuscripts, scientific reviews, oral presentations and regular updates on our websites. This will deter other researchers, pharma and biotech companies from wasting time, money and mice. By becoming Key Opinion Leaders in our respective fields we (Principal Investigators) are already approached by commercial sources for guidance on research programmes and our continued research programme is likely to extend those relationships. In addition my team regularly speak with members of the public through numerous public engagement opportunities that we run and often discuss our need for and justification of animal research. In almost every case the public support our work avidly.

Species and numbers of animals expected to be used

- Mice: 13100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice as a model system to faithfully recapitulate the human disease. These studies will focus on understanding why certain anti-cancer therapies often fail to produce tangible clinical benefits.

Cancer is a disease of organs and tissues, and in essence involves the formation of new aberrant organs called tumours, which are composed of multiple interacting cell types. It is not possible to accurately recapitulate these abnormal organs in culture (i.e. petri dish), and it is only by studying their progression and responses to targeted therapies in animal models that we can learn more of the basic mechanisms of cancer and ultimately design more effective anticancer therapies. Using cells grown in a laboratory does not accurately reproduce all the components that affect tumour development and stages and will not be representative of what could happen in a whole living organism and how it may respond to treatment. For example, we will mainly use adult mice as they will have a fully developed immune system, one of the key features that a tumour 'hijacks' in order to grow and spread, which we would like to study and with the ultimate goal of targeting the 'hijacked' cells. We will also use mice that have been genetically engineered to develop cancer. The types of mutations introduced into the mice are usually designed to resemble human disease stages as our programme seeks ultimately to develop translational therapies i.e. novel treatments that can be given to patients, or improved treatment regimes. It is essential that the genetically altered (GA) animal models faithfully recapitulate the human disease (i.e. stages and response to therapy) to allow us to examine tumour responses to therapies and to identify the molecules (proteins) involved, which ultimately will serve as novel therapeutic targets. In addition, by using mice with inducible genetic changes, that is, genes we can essentially switch on or off, genes that may cause developmental defects (often harmful or lethal in early development), can be regulated by activating the gene(s) in later stages of mouse development, typically during adulthood.

This allows us also to determine whether the gene would be targetable in an adult (eventually adult human) even though it would have been harmful to target in the embryo. Lastly, the use of GA animals engineered to express a molecule of interest specifically in a cell type of interest (i.e. immune cells) are very powerful as they allow us to selectively

target a molecule in a cell type of interest and study its function during tumour development, progression and response to treatment. This knowledge will improve our understanding on how tumour adapt/respond to therapy and will lead to identification of new therapeutic targets.

Typically, what will be done to an animal used in your project?

Typically, cancer cells will be injected into a mouse to allow a tumour to form. Alternatively, some mice have been genetically engineered to develop tumours spontaneously, which faithfully recapitulates the human disease. After a period of time (dependent on the model used, ranging from weeks to months for some slower growing genetic models) the primary tumour may then be removed surgically (under anaesthetic and with pain relief given, according to the advice given from our Named Veterinary Surgeon). Over time (again model dependent) in some of the models metastases (sites of secondary tumours) will form, typically in the lung, liver and bones. We will then be able to image these tumours (under anaesthetic) using modalities such as magnetic resonance imaging (MRI) or bioluminescent imaging (BLI), both standard minimally invasive imaging techniques used widely in animal-based research. Mice will be given different treatments, either as single agents or a combination of agents to try and find the best regimen that could be applied in the clinic. These could be administered via injection or orally. Tumour-free mice (no cancer cell injection) will be used in some experiments to analyse the effects of drugs or biological compounds released by tumours (i.e. extracellular vesicles) in mediating host responses (i.e. inflammation) which are known to pre-condition organs to allow cancer cell colonization and metastasis formation. Blood samples may be taken during the course of the experiment from the tail vein (one drop only removed with limited samples taken) and also at the end of a study where the mouse could have more blood removed under terminal anaesthesia from which they would not be woken. The blood sampling allows us to analyse the effects of a treatment and could help us develop a liquid biopsy applicable to humans, reducing or replacing the number of invasive procedures a patient may have to undergo. At the end of each study all mice are killed humanely and tissues taken for analysis for research. Most studies typically last from days up to 3 months from start to finish, with mouse health being monitored closely taking into account various factors such as appearance, body weight and behaviour.

What are the expected impacts and/or adverse effects for the animals during your project?

Some of the mice used will be genetically engineered to develop a specific type of cancer or disease. As the disease progresses tumour(s) will develop and eventually the primary tumour will spread to other parts of the body. Primary tumours may grow in size to impair mobility but will either be removed surgically (under anaesthetic with pain relief) or the mouse killed humanely before this happens. If a tumour spreads, for example, to the lungs, it may impair breathing but again mice would only experience the minimum discomfort possible before receiving treatment or being euthanised. Some tumours may become ulcerated. In cases where mice present superficial ulcerations, or deep ulcerations that do not exceed the 2mm in diameter, animals will be monitored daily. In cases where deep ulcerations exceed the 2mm in diameter, animals will be euthanized within 24 hours. Some cancers cause weight loss, for which we would provide a softer easier to digest diet. Some therapies or treatments may have some toxicity, for example, chemotherapy, which is also toxic in humans.

Treatments will be administered within ranges that are relevant to patient (mouse) size for the minimum time required possible (usually twice a week, for 4-6 weeks in most cases).

Any weight changes, behavioural changes (hunched posture etc) will be monitored closely. Adverse effects relating to therapies are not expected to reach beyond mild or transient moderate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All mice are anticipated to experience mild (30% of mice) to moderate (70% of mice) severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our goal is to understand the complex biology of cancer and to use this knowledge to develop therapeutic strategies to stop cancer being a fatal disease. To do this we must use systems that match the complexity of human cancer. Cancers are complex organs consisting of multiple cell types which support tumour growth, such as immune cells and fibroblasts,as well as endothelial cells which form blood vessels. Blood supply ensures nutrient delivery to the tumour and removal of metabolic waste, thus supporting tumour growth. Moreover cancer cells use blood and lymphatic vessels to migrate and colonize distant sites where they form metastases.

We use the mouse as an experimental organism for the following reasons: it is an accurate representative of most human organ systems; its diseases show similarities to human diseases; genetic manipulation of the germ line is practical; there is an extensive genetic foundation present in the form of inbred strains of mice and in technologies to map disease loci. We have chosen these preclinical tumour models to truthfully recapitulate the mechanisms that tumors employ to grow. These mouse models will help us to provide useful and reliable insights into the biological mechanisms employed by the tumor to grow, contributing to develop therapeutic drugs with a high potential impact in clinics.

Which non-animal alternatives did you consider for use in this project?

We use the mouse as an experimental organism for the following reasons: it is an accurate representative of human organ systems; its diseases show similarities to human diseases; it enables the growth of experimental tumours that recapitulate the hallmarks of human cancer, including the ability to form metastasis and establish the complex interactions among cancer cells and other host cells, such as vascular and immune cells, which also regulate tumour progression. There are no alternative experimental models (e.g. in vitro assays) that could answer the complex biological questions we wish to address with this project.

However, depending on the scientific question and the nature of cell interactions we wish to study, we will employ co-culture experiments (2D and/or 3D) where possible to reduce the number of animals used in our studies. For instance, we will employ in vitro T-cell mediated cancer cell killing assays, co- culture of primary or immortalized macrophages with cancer cells etc. To study cancer cell invasion and metastasis, we may also use alternative models (if appropriate, i.e. in cases were the molecular mechanism does not rely on complex interactions with complex cellular components such as cells of the immune system) such as the chick embryo assay or zebrafish embyos. Yet, this depends on the complexity and nature (i.e. plasticity, half life) of cells of interest as well as the readout of our experiment.

Moreover, we will perform meta-analysis of publically available datasets from cancer patient cohorts to identify the most clinically relevant mechanisms. For instance, we will seek to identify which signalling molecules or cellular components out of our candidates are associated with disease progression and/or response to treatment in humans, and this will allow us to focus on specific mechanisms and interactions that are most promising to have a translational relevance. This will further reduce the number of in vivo experiments and as such the number of mice used for our studies.

Why were they not suitable?

In vitro systems developed to date can model many disease processes, however they will never fully recapitulate the complexity of a live tissue served by blood vessels and lymphatics and having innate and adaptive immune cells, all of which are key elements of whether a tumour can grow and spread.

Testing anti-cancer drugs in mice in vivo is the only way to assess the importance and relevance of specific interactions between the cancer cells and their stromal microenvironment (including blood vessels, infiltrating macrophages, etc…).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We use statistical programs, applying the unpaired T-test to compare data means or ANOVA with post- hoc paired comparisons, to calculate the differences between expected populations. The statistical significance (or not) of the actual data provides guidance about the minimum number of animals that assure, to the best of our knowledge, a statistically significant outcome. For assessing quantitative effects (e.g., tumor volume; various histological parameters, etc…), our previous experience with the models described here provides guidelines. Briefly, primary tumor growth are relatively variable, so the retrospective statistics give a group size of at least 7-10 mice per cohort/arm in each experiment, depending on the tumor parameters analyzed. Thus, we will evaluate cohorts of 7-10 mice per experiment that should be at the same stage of disease progression or treatment to assure that the results are consistent. We always aim to perform each experiment once. Yet, due to variable factors that may vary between experiments (i.e.

eliminate animals due to tumour ulceration, failure to form tumours etc), or f the results of one experiment are trending toward statistical significance, then the experiment will be repeated with additional mice, aiming to achieve statistical significance, or exclude. However, since tumour response to targeted therapies can be relatively variable (i.e. some tumours may naturally develop resistance while others respond), and we intent to use new combinatorial treatments with unknown phenotype/ response outcome (i.e. some mice may respond, others may be refractory, while others may develop secondary resistance), this may result in classification of experimental animals of a given treatment into subgroups. Inevidably, this will result in reduced animal numbers per subgroup. To study the mechanism of action of the new combinatorial treatments, and only if we observe a trend toward a statistical significance, then new mice will be enrolled until we obtain confidence on the reproducibility of our data and/or a statistical result. Moreover, this number of mice used provides us with sufficient tumor tissue to perform isolation of relatively rare cell types by fluorescence-activated cell sorting and subsequent gene expression studies. We also referred to other relevant published research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experiments will be carried out following good laboratory practice. All experiments will be designed after careful examination of the literature, with regards to treatment i.e. drug dosage, treatment regime and with the aid of the NC3R's Experimental Design Assistant. Whenever possible use of in vitro assays will reduce the numbers of animal required.The minimum number of animals will be utilised for each experiment performed under this Programme of work while maintaining a reliable and measurable output. Sample size calculations will be performed before each experiment so that experiments are adequately powered statistically. Many of these are already done for the experiments planned in this proposal. Using the appropriate numbers of animals is essential to answer reliably the questions we are addressing. This ultimately avoids wasting animals and repeating experiments unnecessarily.

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 maximise the amount of information we obtain from each animal e.g. harvesting as many tissues as possible at the end of study for use for the current and future studies and to share with other researchers when we have fully utilised the samples we need.Imaging technologies will be a vital tool in reducing the number of animals required for each experiment as this removes the need to kill animals at time points to observe tumour progression. To minimise confounding factors we will undertake the following:

experimental design will be well planned with constant checks

within each experiment, animal groups will be maintained under the same husbandry conditions, unless otherwise recommended by the NACWO or NVS

within each experiment, mice will be randomly assigned to different groups

experiments will always be statistically controlled

in some experiments but not all, blinding may be applied (i.e. animal experimentator may use coded drug preparation for the study.

In rare cases, pilot studies will be performed for therapeutic studies where no data are available regarding dosage to reduce the number of mice potentially receiving an ineffective or a dosage beyond maximum tolerated dose (MTD). We will set up the most efficacious breeding programs, with harems to reduce the number of males required and where possible we will breed to produce the maximum number of the genotype(s). For example, if we only require homozygous knockout (KO) mice for our studies we will try to breed homozygous KO male and females to produce only the KO genotype we require, thus reducing the number of unwanted heterozygous or wild type pups that would otherwise be culled.

When reporting on in vivo experiments for publication we will conform to the ARRIVE guidelines and latest NC3R guidelines to minimise unnecessary studies.We will archive genetically modified mouse lines as frozen sperm or embryos to reduce the number of living mice we have to maintain and to allow sharing of these lines between researchers, providing further opportunity for reduction.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Immunocompetent, immunodeficient and Genetically Modified Mice (GEMM) will be used. Some of the GEMM will naturally develop cancers of interest (including breast) so that we can investigate the biology of the developing disease and test our treatments in a model that most closely matches human diseases. Immunocompetent or immunodeficient mice will also be injected with cancer cells (of mouse or human origin) subcutaneously (sc), orthotopically or intravenously (iv), as required by the scientific question, to allow tumour formation. In some cases, tumour-free mice will also be used (untreated, treated with drugs or extracellular vesicles (EVs)). Experienced researchers will inject the animals minimising pain and discomfort to mice.

In most cases, mice do not develop any pain or discomfort as a consequence of their developing cancers and we terminate the animals before such discomfort occurs due to tumour size, or when the general condition of the mouse becomes evidently compromised and signs of distress become obvious. MRI or microCT in vivo imaging of deep tissue tumours provides confidence we can deliver this intention.

In some cases, mice will require anaesthesia and surgery for orthotopic injections and will receive analgesia post-op to minimise discomfort. In some cases, genetically manipulated cells that contain bioluminescence or fluorescence will be used so we can accurately monitor tumour development, metastasis and response to therapy using non-invasive methods so as to minimise pain and harm to animals. All experimental mice are routinely examined for their activity, overall condition and food/water intake. Mice that show signs of pain, distress and/or compromised general conditions will be monitored closely with the local BSU staff and the NVS, and will be humanely killed, as required.

The mice will be immediately removed from the experiment and euthanized if/when they present one of the following humane endpoints:

Compromised ability to walk, eat, drink, urinate or defecate. These functional impairments may occur in a minority mice as a consequence of one of the following: (i) abnormal growth of an individual tumor, which may compromise functions such as locomotion, appetite and/or excretion; (ii) tumor invasion into the chest or peritoneal cavity, which may compromise organ function. Regardless of the ability of the operator in performing tumor cell inoculation, orthotopic or subcutaneous tumors may occasionally invade the chest or peritoneal cavity (orthotopic) or the muscle (subcutaneous).

Difficulty of breathing (dyspnea) possibly caused by metastatic pulmonary tumors.

Persistent hunched or non-responsive to stimuli mouse

Each tumour reaches a volume of 1.25cm3 or becomes ulcerated (>2mm in diameter). Animals will be killed earlier if the tumour ulcerates (>2mm in diameter) or impedes any vital function (e.g. locomotion, mastication, excretion).

Why can’t you use animals that are less sentient?

Most of our experiments require tumours to develop over a time period, which is a result of complex interactions between live cells. These animals will be treated with therapeutic drugs and tumour responses will be monitored/assessed. Thus it is not possible to use animals that are terminally anaesthetised. We use mice as they closely reflect the biological processes in humans that we are trying to investigate. Thus if we were to use non-mammalian models (drosophila, xenopus) we would not recapitulate the biology that occurs in humans. Our choice of mice is the simplest mammal that through years of research by ourselves and others provides us with confidence that our experimental designs are likely to be as close to our therapeutic goals as possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We already are successfully implementing imaging methods at our Institute to improve the analysis and monitoring of deep tissue cancers that occur either through orthotopic implant or grow in transgenic mice. This has improved how we apply experimental therapeutics as we can now 'recruit' mice that have similar volume tumours at Day 1 of a protocol, instead of starting therapy on a cohort at a predetermined time interval after injection. This also allows longitudinal studies that formally required cohorts of mice to be killed at intervals, thus saving many mice. By internal talks and eventually publication we are sharing these improvements with our local and distant scientific community. The imaging protocols (MRI or microCT) have the immediate benefit that there is limited chance that an animal develops an internal tumour that exceeds home office limits. All our mice are kept in individually ventilated cages (IVCs) under barrier conditions, to avoid any infections in immunocompromised mice. Mice are monitored daily for any adverse effects to avoid suffering at all times. We have extensive experience of working with GAA mice so we are aware of health problems and can take timely action to minimise suffering, to ensure pain relief is quickly administered whenever necessary and we have clear guidelines on humane endpoints.

To limit severity in our cancer models we will:

measure tumours regularly to prevent tumours exceeding the legal size limit

determine humane endpoints to produce valid scientific outcomes

image mice to detect unexpected sites of tumour development

use labelled cells to image mice for tumour spread and metastasis

perform pilot studies on orthotopic cancer models to characterise kinetics of tumour growth and metastasis.

perform mammary fat pad orthotopic injections without surgery, under anaesthesia (i.e. isofluorane).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have been following the ARRIVE guidelines and will follow the most up to date version of these that are published by the NC3Rs. We will also make use of the Experimental Design Tool (https://eda.nc3rs.org.uk) and the PREPARE guidelines (https://norecopa.no/media/7832/prepare- guidelines.pdf), as well as the Workman et al guidelines for Cancer research (Workman et al, British Journal of Cancer, 2010).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute regularly hosts updates in experimental animal usage, including developments in 3Rs that I try to attend or send representatives from my team. I and members of my team also regularly attend NC3R events to hear new development on how other researchers conduct their research.

63. Circadian regulation of chronic inflammation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Circadian, Inflammation, Arthritis, Colitis

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to explore how the function of the immune system varies over the course of the 24 hour day, and how these daily changes impact on the development, progression and treatment of chronic inflammatory 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?

Chronic inflammatory diseases such as rheumatoid arthritis (RA), colitis and psoriasis are debilitating conditions. The estimated prevalence of these conditions within Western society is 5-7%. The symptoms of chronic inflammatory diseases often show daily variation. For example, RA patients report increased pain and joint stiffness in the early morning. Similarly, biological markers used in the clinics to assess disease severity fluctuate over the course of the 24 hour day.

The circadian (24 hour) clock is a timing mechanism which synchronises animal physiology to the 24 hour environment created by the earth rotating on its axis. This biological timer regulates numerous aspects of physiology, including sleep-wake cycles,

feeding and metabolism, the gut microbiome, hormone secretion and the immune system. Disruption of the circadian clock, through environmental disruption (e.g. shift work) or through genetic disruption is associated with increased incidence of inflammatory diseases.

The circadian clock plays a critical role in regulating the normal working of the immune system and ensuring appropriate inflammatory responses are mounted when the system is challenged. This work investigates mechanistic links between the biological timing and immune systems to understand the involvement of the clock in regulating chronic inflammatory disease. An ultimate goal of these studies is to reveal novel therapeutic targets or improve existing therapeutic regimes to treat disease through the use of biological timing (chronotherapy).

What outputs do you think you will see at the end of this project?

A major output from this project will be an advance in our knowledge regarding clock control of immune responses. More specifically, information generated from these studies will contribute to our understanding of how the circadian (24 hour) clock affects the development and progression of human chronic inflammatory diseases. This information will further our understanding of how circadian disruption (a consequence of rotating shift-work) may impact on the function of the immune system in health and disease.

It is predicted that data generated by these studies will have a positive impact on the diagnosis and treatment of human chronic inflammatory disease. An example here is the implementation of chronotherapy - timing therapeutic interventions with the peak of disease symptoms. To maximise these positive benefits, it is essential that information obtained from studies outlined here is disseminated effectively. This will be achieved through: publication in academic journals; presentation of data at conferences; and through public engagement events.

A secondary benefit of this programme of work is the advancement of research methods. This may be in the form of refining or enhancing current methods or through the development of new transgenic mouse lines. The data generated from this project will be made available to other researchers in the scientific community at the earliest appropriate time therefore informing further scientific discovery within our research community.

Who or what will benefit from these outputs, and how?

Healthcare sector: There is a growing understanding of the importance of considering circadian time in the diagnosis and treatment of inflammatory disease. This work will further our knowledge of how the circadian timing system interacts with processes underlying human chronic inflammatory conditions. It is hoped that these studies will promote further incorporation of "clock logic" into clinical practice in the long-term. That may be through standardising the time of day at which a patient's blood is sampled for a disease biomarker, or through recommending the best time of day at which to take medication.

Scientific community: This programme of work will generate new research tools and advance research methods which will be shared with the scientific community. Data generated in this project will advance our basic understanding of clock control of immunity and will be of benefit to the wider research community.

General public: The importance of the circadian clock and good sleep hygiene for maintenance of health is becoming widely recognised by the general public. Information obtained through this project will be of interest to people engaging in shift-work and patients suffering with chronic inflammatory disorders. It is becoming more and more evident that disruption of the circadian clock has negative consequences on health. Whilst sometimes circadian disruption cannot be avoided (e.g. shift-workers), for some individuals small lifestyle changes may have a positive impact on health. In order to engage the general public, we will continue to reach out via public engagement events run by the University and charities.

How will you look to maximise the outputs of this work?

Outputs from this work will be published in highly visible journals targeting a multi-disciplinary audience. Through the use of social media (Twitter) and the establishment press office, we will publicise these publications as widely as possible to engage with the general public. In the past, this has led to opportunities to present our research on the radio, television and news websites. We appreciate that it is important to disseminate negative findings to minimise replication of experiments across research institutes. On going work will be presented at international and national meetings aimed at circadian biologists, immunologists and specialised clinicians. To maximise the benefit of our research we will continue to engage with the general public and relevant patient groups through organised events. These activities are important for maintaining public interest in our research and also further developing trust in UK research.

Species and numbers of animals expected to be used

- Mice: 24 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These studies will utilise juvenile and adult mice, which may be genetically modified. Mice are the most appropriate species for these studies as the systems which we are studying here (the body clock and immune system) are well reproduced between mice and humans.

Typically, what will be done to an animal used in your project?

Animals may be monitored non-invasively for behaviour under normal or altered environmental conditions (typically for periods lasting 2-6 weeks). These environmental manipulations include changes in the light/dark cycle, changes to the composition of the diet or changes in timing of food availability. More rarely, animals may undergo physiological monitoring using implanted telemetry devices (requiring a brief surgical procedure) or imaging facilities (under recovery anaesthesia). Rarely repeated imaging may be utilised (up to 8 times in one day). Changes to rhythmic biological signals (such as hormones or microbial metabolites) may be instigated through implantation of hormone pellets under the skin (brief surgical procedure); through application of antibiotics; or through administration of a chemical reagent. On occasion when utilising specific strains of

transgenic animals a gene inducing agent may be administered to the animal in order to "switch off" the gene of interest.

To instigate chronic inflammation different approaches will be utilised, each modelling a different human chronic inflammatory disorder. In each instance, mice will usually only be maintained in a chronic inflammatory state for 5-10 days. Each of these models will be used alone (never in combination).

Joint inflammation: This may be induced via an injection of collagen which drives an autoimmune response and chronic inflammation of multiple joints (collagen induced arthritis, CIA) or via use of methylated bovine serum albumin which drives a resolving inflammatory arthritis affecting just one joint (antigen induced arthritis, AIA).

Gastrointestinal inflammation: This may be induced via temporary administration of a chemical agent in the drinking water which causes damage to the gut barrier. This drives inflammation of the colon which resolves over time when the chemical agent is withdrawn.

Skin inflammation: This may be induced via application of an agent to the skin of the ear or the back, which causes a resolving local inflammatory response.

Prior to, or during these procedures to induce an inflammatory response, the immune system may be manipulated. This may be via application of reagents to target specific pathways. On rare occasions the immune system may be manipulated further through irradiation to deplete host immune cells before replacement with donor cells. Additionally, reagents may be administered which target the clock or the immune system to establish their effects on inflammatory processes.

Animals utilised in these models of inflammation may be assessed using *in vivo* imaging, collection of small volume blood samples and/or assessment of metabolic function or gastrointestinal function. To assess metabolic function, animals may be administered an injection of insulin or glucose and blood samples taken, or may have harmless metabolic tracers added to the drinking water. To assess gut barrier permeability animals may be orally administered a fluorescent labelled sugar prior to collection of small volume blood samples.

In some instances, a line of mice will be utilised which express a green fluorescent protein which can be converted to a red fluorescent protein through application of violet light. Here, animals may be briefly anaesthetised and exposed to this light source in order to be able to track the movement of cells from one site in the body to another.

In addition to these procedures, mice will be used in this project for breeding, and for provision of cells and tissues for *ex vivo* studies.

What are the expected impacts and/or adverse effects for the animals during your project?
Animals may experience mild adverse effect such as temporary stress or brief pain and discomfort. Temporary stress may be induced by a brief period of restraint (e.g. in order to administer an injection) or alterations in their housing environment (e.g. single housing). Mice may experience a brief period of pain and discomfort in response to dosing (e.g. injection or oral gavage) or blood sampling (e.g. tail bleed) or following surgical intervention to implant a telemetry device or hormone pellet or to apply a photoconverting light source. In these instances the stress and discomfort will be transient.

Weight loss may occur after manipulation of the diet, but will be transient and will either reverse or stabilise. Transient dehydration and weight loss may occur as a consequence of addition of an agent to the drinking water which may make it less palatable or as a consequence of irradiation protocols.

Models of chronic inflammation will induce localised inflammatory responses resulting in pain. Models of arthritis (collagen induced arthritis (CIA) and antigen induced arthritis (AIA)) will lead to swollen, inflamed joints and may results in reduced mobility. CIA is a polyarthritic model whereby multiple joints may be affected. Animals are usually maintained in an arthritis condition for 7-10 days. Affected animals exhibit reduced activity and may exhibit some weight loss. AIA is a monoarthritic model of resolving inflammatory arthritis whereby only one knee joint is affected. Here, animals are usually maintained in an arthritic condition for up to 10 days. These animals may exhibit a brief period of reduced activity (1-2 days) and rarely exhibit weight loss. On occasion (<20% AIA animals) mice may be manipulated to induce one or two subsequent "flares" after the initial insult in order to model periods of increased disease activity experienced in human disease.

Gastrointestinal (GI) inflammation is associated with pain, development of diarrhoea, occasionally the presence of blood in the stools and weight loss. The inflammation inducing agent is withdrawn after a period of time (usually 5 - 7 days) after which the inflammation resolves. On occasion (<20% colitic animals) mice may be manipulated to induce one or two subsequent "flares" after the initial insult in order to model periods of increased disease activity experienced in human disease.

Skin inflammation is associated with reddening, scaling and thickening of the skin. This is associated with pain and discomfort and weight loss. Here animals are usually maintained in this condition for 5-10 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The studies outlined in this project will result in a cumulative impact to the animals (mice) that are sub- threshold to mild (approximately 60 percent) or moderate (approximately 40 percent) severity rating.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In the context of organ-level inflammation, it is currently not yet possible to replicate the complex multi- cell environment underpinning disease. Thus, *in vitro* cellular models and *in silico* models have limited application as a replacement for studies of chronic inflammatory

disease. In order to study complex interactions between the circadian timing system and immune responses, mouse models are the most appropriate approach.

Which non-animal alternatives did you consider for use in this project?

In vitro approaches include the use of single-type cell lines or co-culture systems (where two or more relevant cell types are studied together). We utilise these methods to inform the direction of animal studies. *In vitro* assays can be used to test how genetic or pharmacological interventions alter the function of the cell intrinsic clock and/or regulation of inflammatory processes. For example, *in vitro* assays using cell lines or primary cells allow us to first test a diverse range of potential therapeutic interventions and identify a small number of candidate molecules with the most potential for subsequent use *in vivo*. However, these approaches cannot replicate the complex environment of the joint, gut or skin and cannot replace the use of animals.

This programme of work is supported by parallel studies using tissue samples from humans. Synovial fluid and serum collected from healthy volunteers and rheumatoid arthritis patients will be analysed to assess time of day variation in inflammatory mediators and metabolites. Additionally the group routinely utilises online databases such as UKBiobank and NIHR IBD Bioresource to examine circadian features of patients with chronic inflammatory disease.

Why were they not suitable?

It is critical that any cell lines utilised possess a functional clock. Our experience is that a cell type may be rhythmic in a healthy animal, but once removed and cultured it becomes less rhythmic. This may be due to the absence of other signals (for example hormones) that these cells would normally be exposed to. Furthermore, *in vitro* assays cannot adequately model the complete array of inflammatory responses or address how systemic timing signals may modify these responses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?
Our grouping has extensive experience with the methods and approaches outlined here and of running projects of a similar scope. Consequently, estimates of animal numbers are based on previous experience and with careful consideration of the experimental design. Where protocols are new to the group, we will run small control experiments (taking advice from local colleagues with expertise in these models) to generate data on which to perform power calculations in order to plan future studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When considering the design of experiments underpinning this project we have consulted with statisticians to gain specialist advice on the types of experiments that will be

undertaken and the nature of the datasets that will be collected. We will continue to do this as the work develops. We will be utilising purpose written software (such as the NC3Rs Experimental Design Assistant) to further support experimental planning, randomisation and blinding.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Throughout this project we will continue to optimise animal use wherever possible. This starts with efficient breeding of transgenic mouse lines, which we achieve through close monitoring (facilitated by specialist colony management software) and effective communication with technical staff managing the colonies. We aim to minimise numbers of animals bred whilst still achieving adequately powered, age and sex-matched groups of experimental animals. When individual projects have been completed breeding of relevant mouse strains will be minimised until a suitable time to preserve the colony by freezing down gametes.

Pilot studies are utilised to optimise experimental conditions when we are developing new approaches. Where it is appropriate we utilise technologies that permit longitudinal assessments in the same animal. Furthermore, we always look to maximise the amount of data that we can gather from a single sample using the latest technologies to their full capacity.

At the end of each experiment we carefully consider which tissues to collect with future studies in mind. By building a well archived tissue bank we are able to utilise existing samples in the laboratory to test new protocols or reagents without the need to utilise further animals. We make our banked tissue available to our collaborators. Through providing the wider scientific community with access to data generated through our studies (through online data repositories) we maximise the scientific knowledge than can be obtained from our animal studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A substantial proportion of the methods that we will use in the project are non-invasive and involve simple environmental manipulations to study effects on circadian physiology and/or immunity. This includes modification of the lighting, meal composition or meal timing. These manipulations are unlikely to cause suffering or distress, however occasionally may require an animal to be singly housed. To minimise potential distress in this situation, where possible we will supply mice with environmental enrichment (e.g. plastic tubes, wooden logs or nestlets). Some animals may be subject to a brief surgical procedure, for example to implant a device which permits remote monitoring of mouse activity.

Where we seek to administer reagents to experimental animals we utilise the most refined route of administration possible. For example, we routinely administer antibiotics in the drinking water, rather than through oral gavage. In order to make the solution of antibiotics more palatable to the animals we add a sweetener. We always seek to minimise the numbers of doses of a treatment in order to achieve our objective.

Pre-clinical models of human chronic inflammatory disease will be used in this project, including inflammatory arthritis and models of gastrointestinal inflammation and skin inflammation. We have chosen the most refined models available to address our experimental objectives. For example, we no longer use the K/bXN model of arthritis (in which mice spontaneously develop severe erosive arthritis from an early age). With each of these models, we restrict the period of inflammation as much as possible to minimise animal pain and suffering without compromising experimental aims.

Why can’t you use animals that are less sentient?

We cannot replace these studies in mice with studies in another species (such as insects or fish) to achieve our objectives, as they lack the complex immune and circadian systems seen in higher order species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We routinely seek to minimise stress and discomfort to animals during our work and achieve this by ensuring all researchers utilise appropriate animal handling techniques (e.g. tube handling where possible to remove animals from their cage) and by using environmental enrichment where possible, especially in instances where it is necessary to singly house animals.

Occasionally animals may undergo a brief surgical procedure. After surgery, animals are closely monitored and post-operative care provided (pain management with analgesics and provision of extra fluids). Analgesics may be provided in the form of a palatable gel to encourage voluntary ingestion.

Animals which develop chronic inflammation are monitored regularly, including monitoring weight loss and general condition. Analgesics may be utilised in animals exhibiting chronic inflammation, however the use of pain relief is not always compatible with our experimental aims. Where transient weight loss is expected as a consequence of the experimental condition, soft food (wet mash) will be provided on the cage floor as well as environmental enrichment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure that our experiments are conducted in the most refined ways we continually assess our experimental design and re-assess approaches if the opportunity arises. We stay informed about best practice guidelines by referring to information provided by Laboratory Animal Science Association (LASA) and NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our group stays informed about recent advances in 3Rs approaches by staying up to date with NC3Rs recommendations and developments. This information from the NC3Rs is obtained through interaction with their website, local seminars, contact with their staff and through social media (Twitter). We endeavour to adopt new tools as they evolve, such as the NC3Rs self assessment tools, in order that we focus our efforts here and keep up to date with successful initiatives utilised elsewhere. We also discuss further refinement opportunities with our NVS and NACWO and through interaction with colleagues at conferences, workshops and seminars.

64. Investigating new radiotherapy and drug treatments 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, radiotherapy, chemotherapy, radiation

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop mouse models of cancers that are difficult to treat, and use these models to identify new treatments that can increase the effectiveness of radio and chemotherapy. We will also use the models to understand why some tumours are less responsive to radiotherapy or chemotherapy. Finally, we will investigate the effects of radiation on normal (non-cancerous) tissues with a view to reducing the side effects of radiotherapy treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

These studies are essential because many cancers are resistant to current treatments, which often include radiotherapy and chemotherapy. The proposed work will enable us to develop and test new radiotherapy and radiotherapy-drug combinations to treat cancer

more effectively. We will also study ways to reduce the adverse effects of radiation on normal tissues, in order to reduce harmful side effects of radiotherapy.

What outputs do you think you will see at the end of this project?

We will develop mouse models of cancers that are currently difficult to treat, including brain tumours, lung cancer (including mesothelioma), and head and neck cancer, and use these models to identify new treatments that will increase the effectiveness of current treatments, particularly radiotherapy and chemotherapy. We will also use the models to understand why some tumours are less responsive to radiotherapy or chemotherapy than others. Finally, we will obtain new information on how radiotherapy damages healthy (non-cancerous) tissues, and find out if certain drugs can reduce the extent or severity of this damage. Our studies will be designed so that they can lead directly to clinical trials. We will publish our results in high-impact scientific journals so that other researchers will have access to our models and our data. Experiments aimed at refining and improving our mouse models of cancer will create new and better models that are likely to give more accurate results. In parallel, further development of 3-dimensional cell culture models will enable more efficient evaluation of new cancer treatments while minimising the use of mouse models.

Who or what will benefit from these outputs, and how?

The benefits of this project are:

A more in-depth understanding of the biology of cancers that are difficult to treat, including brain tumours, lung cancers (including mesothelioma) and head and neck cancers.

Better understanding of why these cancers are resistant to current treatments, including radiotherapy and chemotherapy.

Accurate testing of new therapies for cancers that are difficult to treat; this will identify which treatments that are most likely to be successful in the clinic.

Improved understanding of the processes responsible for the side-effects experienced by patients undergoing treatment with radiotherapy or radiotherapy-drug combinations.

Improved understanding of the side-effects of radiotherapy and radiotherapy-drug combinations experienced by animals undergoing treatment; this information may help us find was to reduce side-effects experienced by both animals and humans.

Ultimately we aim to improve outcomes for patients with cancers that are difficult to treat by developing more effective treatments and reducing side-effects.

How will you look to maximise the outputs of this work?

To maximise the outputs of our work we will continue to work closely with academic and industrial partners across the UK and internationally, and continue to establish and lead working groups in the field of radiotherapy-drug combinations. New knowledge will be published in high impact scientific journals and presented at national and international conferences. In particular, we will continue to work with our networks of scientists and clinicians to ensure that the most promising treatments are tested in cancer patients as soon as possible. Unsuccessful approaches will also be published to add to the body of knowledge and minimise the chance of these approaches being tested by other

researchers.

Species and numbers of animals expected to be used

- Mice: 2650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using genetically altered mice or mice without an intact immune system that carry specific mutations in genes that are thought to play a role in cancer. This includes genes involved in the response to tumour development and response to treatment, including changes in the immune system or metabolic processes.

Genetically altered mice can model the genetic mutations of human cancers and allow us to look at the effects of these genes in normal non-cancerous tissues, as well as studying how cancers develop. We can observe the effects of gene alterations within the mouse host and their effects on tumour growth and response to treatment.

Mice do not have an intact immune system enables us to transplant human tumour cells and some specific types of mouse cells into the host mouse without rejection by the body. This is extremely important because it allows us to study cancers that grow in patients, and to test new treatments on these 'human' cancers. Transplant models also allow us to manipulate the genetics of distinct cell types (either the cells of the mouse host or external transplanted cells), to examine their impact on responses to anti-cancer therapy.

Most of our experiments will be conducted in young mice (typically aged 8 - 10 weeks at the start of the experiment). In some of our cancer models (either genetically engineered or transplanted) it can take many weeks or months for tumours to develop. For some experiments we will use adult animals (typically aged around 6 months at the start of the experiment) for two reasons: (1) cancer is more common in older people so adult mice can provide a more relevant environment, (2) some of the side- effects of treatment are more common in older people, so these can be studied more accurately in adult mice.

Typically, what will be done to an animal used in your project?

Cells from patient tumour samples or commercially available established cell lines will be injected into the animal to create cancerous tumours of the types being studied. Tumour cells may be injected into the brain (under general anaesthesia), under the skin (without anaesthetic if cells, with anaesthetic if tumour fragments) or both.

Radiotherapy may be administered (under general anaesthetic) and its effects on tumours and healthy non-cancerous tissues will be determined by imaging and/or by taking a tissue biopsy or sample. We will use established and emerging imaging and tissue analysis techniques to gain the most data from each animal. Some mice will have a perspex 'window' inserted into their skull (under general anaesthetic)- this will enable us to obtain detailed, real-time information about tumour growth and the effects oftreatments on cancers growing within the brain.

Mice may be treated with other anti-cancer therapies, either before or after radiotherapy, to investigate the effects of radiotherapy-drug combinations on tumours and on healthy non-cancerous tissues.

Therapies may be given before tumour injection, or at early or late stages of tumour development provided the animal is in good health.

Drug treatments may be given by mouth or by injection (without anaesthetic), depending on the type of drug.

Animals may be imaged non-invasively, using techniques such as ultrasound, CT, PET or MRI scans, pre- and post-treatment as a means of assessing the effects of treatment on tumours and healthy tissues. All imaging procedures are performed under general anaesthetic.

Experiments using the intracranial tumour model will generally last between 10 and 24 weeks. Tumours become detectable around 10 weeks after intracranial injections and studies of drug distribution and acute effects of radiotherapy and/or drugs will be completed within 12-14 weeks. Experiments studying the effects of treatments on long term tumour growth will be completed with 16 and 24 weeks, depending on how effective the treatments are.

Experiments using subcutaneous tumours will generally last between 2 and 12 weeks, depending on how rapidly the tumours grow and how effective the treatments are.

What are the expected impacts and/or adverse effects for the animals during your project?

Adult animals are susceptible to diseases of old age such as lumps and masses, eye problems, decreased muscle mass, and reduced strength and endurance resulting in loss of mobility, altered gait or reluctance to move.

Blood sampling can cause bruising (<5%), bleeding (<5%), or infection on very rare occasions (<1%).

Injections can result in momentary discomfort (100%) at the time of injection by the needle. In rare cases injections can cause tissue damage and infection (<1%), e.g. intravenous injection has a risk of vein damage.

Fasting may result in mild, transient stress.

The commonest side-effects of anti-cancer drugs are a decrease in white blood cells (<10%), anaemia (<10%) or gastrointestinal issues that cause weight loss or diarrhoea (<10%). These side effects typically last a few days.

Radiotherapy can cause different side-effects depending on the part of the animal being treated. The most common side-effects are hair loss (<50%, lasts several days), diarrhoea (<10%, lasts a few days), weight loss (<10%, lasts a few days) or increased respiration rate (<5%, lasts a few days).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All protocols have a moderate severity rating. All animals (100%) on protocols 1-5 will experience a moderate severity rating.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although many studies can be conducted using cells growing in the laboratory, they cannot fully model the complexities of cancer development in the body. It is well-known that the immune system and other bodily processes play an important role in cancer growth; these complex processes cannot be recreated in cell culture. It is also known that the effects of radiotherapy on tumours and healthy tissues are strongly influenced by the surrounding tissues and by the immune system. To our knowledge there are currently no non-animal alternatives that fulfil the criteria required for our experiments; this has been investigated using the website www.frame.org.uk.

Most new treatments developed in the laboratory have failed to provide benefits for patients; an important reason for this is that laboratory studies have failed to recreate accurately the immune system and other bodily processes which are important in the development of the cancers we are studying.

Which non-animal alternatives did you consider for use in this project?

We have considered laboratory based cell culture systems as alternative models, but these do not encompass the complex interactions between cancer cells, healthy tissues and treatments that occur in living organisms.

To address this, we have developed 3-dimensional cell culture systems that incorporate some of the features of the tumour microenvironment and can be used for some of the experiments in our research programme. This is reducing our use of animals. However, these 3-dimensional models are not available for all cancer types, and do not include all the necessary features of living organisms, so we still need to use mice for many of our experiments.

Why were they not suitable?

Cultured cancer cells show many structural and biological differences from cancer cells growing in intact tumours; these affect their growth and metabolism and also affect their response to treatment. There is increasing awareness that observations made in cancer cells growing in the laboratory differ from those made in cancers growing in live organisms. In particular, drugs and treatments such as radiotherapy that are found to be

effective in cancer cell culture systems are very often ineffective in the clinic.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated that across the six protocols included in this project we will use approximately 2650 animals over the five year period of the licence. Approximately 1250 of these mice will be used in studies of tumours growing within the mouse brain, since this is the major focus of our laboratory.
Approximately 100 mice will be used in cranial window model studies - numbers are fewer than the other protocols because longitudinal data is obtained at multiple timepoints from each mice, so much smaller group sizes are needed. Approximately 500 mice will be used for studies involving subcutaneous tumour models; these will mainly (but not exclusively) involve studies of lung cancer (including mesothelioma) and head and neck cancers. Approximately 300 mice will be used in studied evaluating blood brain barrier penetration of therapies; smaller numbers of mice are needed for each of these drug delivery experiments than for efficacy studies, but we aim to evaluate multiple different drugs in this way over the period of the licence. Approximately 500 mice will be used for studies evaluating the effects of radiotherapy on key normal tissues.

The numbers of mice needed for each experiment have been estimated based on previous work in our laboratory and in the laboratories of our collaborators and partners. We have established the degree of variability of the models used and are therefore able to predict the numbers of mice required to obtain robust data. We have estimated the total number of different drugs and treatments that we aim to evaluate over the period of the licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Pilot studies with 3-4 mice will always be performed when using new cell lines and therapies, and small studies to measure drug levels in brain tumour models will always be performed before larger studies. Data from the pilot studies will be used to determine how many mice are required to accurately assess the effectiveness of the new treatments.

For experiments involving tumours growing within the brain, we will start with small studies that measure whether drugs are penetrating the tumours at sufficient concentrations. before proceeding with larger experiments to see if the treatments are effective. If a drug does not penetrate the brain tumour sufficiently, we will not proceed with further experiments.

Where possible, we will use non-invasive imaging methods to monitor tumour growth and responses to therapy. This will permit studies of tumour growth over several weeks, in the same way that tumour growth is monitored in humans, and will avoid unnecessary sacrifice of animals at different time points. Obtaining imaging data at multiple timepoints increases the amount of information obtained from each mouse and often reduces the number of animals required for each experiment.

The NC3Rs online Experimental Design Assistant and G-Power tool will be also used to guide study design and calculate sample sizes required for quantitative experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Laboratory based testing in conventional 2D cell culture and more representative 3D cell culture models will be used to identify promising treatments prior to moving on to animal experiments. Pilot studies using 3-4 animals will always be carried out prior to proceeding with larger studies. These studies enable identification of adverse effects on animals, refinement of treatment protocols if necessary and determination of whether full-scale experiments to measure effectiveness are justified. Pilot studies also help us to answer scientific questions more efficiently.

Wherever possible, we will use non-invasive imaging methods to monitor tumour growth and responses to therapy. This will permit studies of tumour growth over several weeks, in the same way that tumour growth is monitored in humans, and will avoid unnecessary sacrifice of animals at different time points. Obtaining imaging data at multiple timepoints increases the amount of information obtained from each mouse and can reduce the number of animals required for each experiment. Tumours will be excised at the end of each study and assessed by molecular and/or histological techniques. We will preserve tissues for future research use and create sample databases that can be shared with other researchers. Other researchers in our Institute are developing non-vertebrate models of cancer (e.g. Drosophila (fruit flies) and mathematical models), which although limited at present are hoped to be expanded to many tumour types over the next five years.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The following animal models will be used during the project:

The intracranial tumour model will be used because it reproduces key characteristics of brain tumours including their interactions with the very specific environment of the brain. These interactions, and this environment, play crucial roles in determining the way in which brain tumours respond to treatments such as radiotherapy and drugs. It is therefore necessary to test new treatments for brain tumours in the intracranial tumour model. It is now well established that subcutaneous models of brain tumours generate very misleading results. By using the intracranial tumour model we will avoid unnecessary use of mice in misleading experiments.

The cranial window model allows live imaging at a microscopic level of tumour cells growing within the brain - without killing the mouse. The microscopy technique used to

visualise the brain provides the highest achievable resolution of live tumour cells in the animal, and imaging at multiple timepoints generates scientifically rich datasets that significantly reduce the number of mice needed for each experiment.

We will use subcutaneous tumour models when studying cancers that grow outside of the brain. For example, subcutaneous models recapitulate many of the key features of head and neck cancers, which arise in mucosal surfaces of the head and neck region. We will also use subcutaneous where orthotopic models are either unavailable, fail to reproduce the key feature of the cancer being studied, or are not amenable to radiotherapy. For example, many genetically engineered models of lung cancer generate diffuse or multifocal tumours that can not be localised for radiotherapy treatment. By ensuring that our models are as clinically relevant as possible we will minimise unnecessary use of mice in misleading experiments.

For all studies that require radiotherapy, we use a Small Animal Radiation Research Platform that incorporates a CT scanner and allows extremely accurate irradiation of tumours with 0.5mm precision. This greatly reduces the amount of tissue exposed to radiation and minimises the side-effects of treatment.

Why can’t you use animals that are less sentient?

It is essential that we use mice in order to model the genetic mutations of human cancers and allow us to look at the effects of these genes in a normal non-cancerous body and how the cancer develops over time. We can observe the effects of gene alterations within the mouse and its effects on tumour growth and response to treatment in a similar way to that in the human. It is not possible to implant human tumours in animals at a more immature life stage, and species that are less sentient do not possess the complex tissue architecture that is required to study tumour growth and development, and to enable measurement of the effects of treatments. Since tumour growth and response to treatment takes several weeks or months, it is not possible to perform these experiments in animals that have been terminally anaesthetised.

We will be using adult animals because cancer is a disease of ageing and some types of cancer take many weeks or months to develop into tumours.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pilot studies using 3-4 animals will always be carried out initially when assessing new agents to minimise the adverse effects on animals. Pilot studies will also be performed when using new tumour cell lines to determine the take rate and for characterisation of tumours. This will determine if a full-scale experiment is merited and will help answer scientific questions efficiently.

The use of non-invasive imaging will allow a number of data points to be acquired from the same animal, therefore reducing the number of animals used. During imaging, the body temperature of the mouse will be maintained and regulated to reduce potential harms caused by body temperature stress.

Our methods are refined and monitored to ensure the minimum pain and distress for the animal. All injections cause momentary needle-stick pain. Repeated injections on the same day are avoided. Only experienced handlers will carry out injections and they will use aseptic technique, fine gauge single use needles, good restraint, small volumes and

slow delivery. Intracranial tumour implantation is performed under general anaesthesia and pain and distress are further minimised by the use of analgesics and antibiotics.

Administration of therapeutic agents in the diet may cause unpalatability so mice are weighed for the first week after introduction of a new diet and those with 15% reduction in weight, compared to matched controls, will be returned to normal diet.

Animals will not be imaged more than 30 times (once per week for up to 30 weeks) using PET, fluorescent, bioluminescent or ultrasound modalities. For MRI scans, animals will be imaged no more than 10 times with a recovery period of 24hrs between scans.

All surgeries will be performed in a dedicated surgical suite and always using aseptic techniques and sterilised surgical instruments. Pre and post-operative pain relief, and anti-microbial therapy (for the cranial window model only) will be administered routinely to animals under-going surgery as advised by the NVS and in line with current guidelines. Mice undergoing surgery will be monitored closely during the surgery and frequently afterwards by fully trained staff. Animals will be maintained in a warm environment until recovery from anaesthetic.

For studies that require radiotherapy, equipment incorporating a CT scanner is used, which allows extremely accurate irradiation of tumours with 0.5 mm precision. This significantly reduces the amount of tissue exposed to radiation and hence reduces the likelihood and severity of normal tissue toxicity.

To minimise suffering, all mice are frequently monitored and humanely killed when exhibiting signs of altered health status and/or tumour burden. All users will be fully trained in monitoring tumour development for each model and will be signed as competent prior to initiating their studies. Any animals exhibiting 3 signs of moderate severity, or a single substantial severity will be humanely killed; however, other methods of monitoring e.g. the grimace scale and body condition scoring, will also be explored and adopted in to our routine monitoring if they prove useful.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all of our studies we will refer to the Guidelines for the Welfare and Use of Animals in Cancer Research (Workman et al, 2010) and ensure best working practice. We will consult the NC3Rs guidelines, including any updates or amendments, and monitor refinement where such practices are published (www.nc3rs.org.uk).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All our laboratory staff are fully trained and experienced in the experimental work undertaken. They are required to provide proof of training and take part in continuous professional development including updates in the 3Rs and any published advancements in the field. This is assessed on an ongoing basis by senior staff. Implementation of any advancements or amendments to best practice is overseen by senior staff to ensure the laboratory team are working to the most recent guidance. Having received funding from NC3Rs in the past, we are on their mailing list and have access to their online resources such as webinars.

One of the many benefits of working within consortia is access to new models, techniques

and resources that have been developed by other members of the group. We are members of a national Working Group that promotes and facilitates the development and sharing of advances in the 3Rs for radiotherapy researchers.

65. Mapping mechanisms for energy homeostasis in rodents: communication between brain and peripheral tissues via the action of fuel sensing protein kinases

Project duration

2 years 0 months

Project purpose

- Basic research

Key words

Diabetes, Obesity, Hypothalamus, Energy, Homeostasis

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate how fuel sensing in a region of the brain, the hypothalamus, influences energy homeostasis via action in peripheral tissues.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The regulation of energy intake and expenditure is of high importance in health and in disease states such as type 2 diabetes (T2D) and obesity. Control of energy intake (e.g. by the ingestion of food) and energy expenditure (e.g. mobilisation of stored fuel for the fight or flight response) are coordinated and regulated by the brain, in brain centres like the hypothalamus. Metabolic diseases like T2D and obesity, manifest when energy intake and expenditure are out of balance, leading to the symptoms seen in patients, which in turn leads to a decrease in the quality of life of the patients. While we have a good understanding that the hypothalamus is involved in the control of energy balance, it is not

currently clear how the hypothalamus detects the changing levels of fuel (i.e. nutrients) in the body over time in order to achieve this balance. It is well documented that some proteins act as fuel sensors and their function is to detect changing levels of fuel, leading to changes in cellular function to deal with low or high fuel situations.

The core focus of this project is to investigate the role of the novel cellular fuel sensor (PAS-domain containing protein kinase; PASK) in the detection of nutrient status to control energy use in the hypothalamus. Our preliminary data indicate that PASK may be a target for more effective anti-diabetic and anti-obesity drugs, so the studies in this project will allow us to map how PASK works in the hypothalamus and, in the longer term, to inform future drug development.

What outputs do you think you will see at the end of this project?

At the end of this project we will have answered to the key question that we posited in our Aim: whether or not PASK in the hypothalamus is important for nutrient detection in the hypothalamus.

We will disseminate this information to the scientific community and to the public. We will publish our findings in peer-reviewed journals and disseminate this information to the scientific community at national and international conferences. We will disseminate our findings to patient groups and other interested stakeholders via our outreach programme.

Who or what will benefit from these outputs, and how?

In the short term, colleagues and other researchers in the field will benefit from the data we will obtain concerning the function of PASK in the hypothalamus.

In the medium term, the data will be published, meaning that the wider research and clinical communities will have a greater understanding of the mechanisms of nutrient detection and energy control.

In the long term, beyond the lifetime of this PPL, the project outputs will lead to further funding applications to perform research on drugs that regulate PASK function, which may impact upon patients through the development of novel therapeutics for adaptive energy control in diabetes and obesity.

How will you look to maximise the outputs of this work?

This work will generate new knowledge which we will disseminate through publication of the data and presentation of our findings at national and international conferences. We will publish the data even if the data should be negative in peer-reviewed scientific journals and/or in preprint so as to prevent repetition of the work.

Species and numbers of animals expected to be used

- Mice: 260

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice because the project aim focuses on mapping function within a complex system of nutrient detection and energy control. Because of the necessary relevance to human health and disease, we need to study these mechanisms in mammalian systems. Our rationale is the following:

While lower organisms can model individual elements of the mammalian system, the focus on energy intake and expenditure at the whole body level can only be conducted in live mice. Mammalian systems are required , as although lower organisms such as zebrafish are emerging as potential models in which to study metabolic diseases, they do lack some of the important modulators of energy regulation of mammals- e.g. brown adipose tissue.

Mice have previously been used to elucidate the involvement of PASK in the control of feeding behaviour and whole body energy balance. These studies have allowed us to elucidate the involvement of PASK in the regulation of energy homeostasis in peripheral tissues (like in fat and in the pancreas), but not PASK's involvement in the control of food intake and other energy balance mechanisms which are controlled principally by the brain e.g. in neurons in a brain region called the hypothalamus. We wish to use the same species for the continuation of this work as the data already gathered can be used to inform future project direction.

Because of the need for experimental approaches to isolate the mechanistic targets of interest, we cannot use human studies. The use of live animals is necessary because there are no appropriate alternatives and the timescale of effects spans over many days.

We will use adult mice because energy expenditure and resources change during development, and our aim is to study adult physiology.

Typically, what will be done to an animal used in your project?

Typically mice will be acutely injected with viruses in the brain to allow manipulation of PASK gene expression in specific neurons in the brain, which we predict (based on our preliminary darta) will lead to energy imbalance. To assess the effects of altered PASK gene expression, we will measure food intake and energy expenditure using non-invasive procedures which will take such measurements in an automated manner, i.e. reduce stress from handling. The ability of the mice to deal with glucose balance will be assessed by a glucose tolerance test which involves fasting mice, then giving them a sugary solution and measuring how quickly the glucose is cleared from the blood (as we do on patients with diabetes).

What are the expected impacts and/or adverse effects for the animals during your project?

Injection of the viruses in the brain will involve surgery. Following surgery, mice will feel pain and discomfort where the skin has been sutured (typically for up to 3 days). This can be accompanied by some weight loss or failure to gain weight (which returns to normal within 7 days). Mice can occasionally display signs of minor discomfort during administration of substances into the brain (which can last for up to 2 min). These signs do not last beyond the duration of the injection. Mice can experience stress as a result of the fasting for the glucose tolerance test.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals anticipated to experience a moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The successful balance between energy intake and use requires the interplay between many parts of the body such as the pancreas, the liver, the skeletal muscle and adipose (fat) tissues. Signals from the brain in response to changes in hormonal signalling and nutrient availability also modify important factors such as blood glucose concentration and feeding behaviour. Such complex interrelations cannot be reproduced in vitro and require a whole living organism. We make efforts to replace the use of rodents for our studies as much as possible. We conduct a lot of our work in vitro using cell lines and cell cultures. Thus, we are able to validate fundamental mechanisms in vitro, before evaluating their relevance to physiologically-relevant energy intake and use processes in awake behaving animals.

Which non-animal alternatives did you consider for use in this project?

I considered using neuronal cell lines or hypothalamic slice cultures.

Why were they not suitable?

Neuronal cell lines or hypothalamic slice cultures do not allow us to monitor feeding behaviour, effects on weight, and control of blood glucose.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based the number of animals to use on published data from other laboratories and from the experience of the PPL holder in these types of experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3R's Experimental Design Assistant to help us to design the experiments to ensure that the experiment uses the minimum number of animals but that it is sufficiently powered to achieve our scientific aims. The design is also guided by published data from similar types of experiments. We have tested the efficacy of the viruses in vitro so that we have confidence in them functioning as designed when used in mice.

To monitor energy expenditure, we will place mice in specialised cages that will allow us to monitor various parameters non-invasively and continuously. This will replace requiring regular handling by/presence of an investigator to make these measurements at regular intervals. This will mean needing to use fewer animals than if we had to collect samples/tissues time point by time point in order to get this information indirectly.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The efficacy of the viruses have been validated in vitro so that we have confidence in them functioning as designed. Experiments will be planned so that they can be published in accordance with the ARRIVE 2.0 guidelines. At the end of the experiment, we will harvest the maximal possible number of tissues. Tissues not immediately analysed will be archived and will be made available to other researchers working on similar questions.

We will use Cre-strains such that the genetic manipulation will be targeted only to cells that express Cre-recombinase in the animal. This will limit the effects of the genetic manipulation only to the sites that we are interested in probing, minimising off-target effects, and also lowering the animal numbers needed (due to a decrease in experimental noise from off-target effects).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To monitor energy expenditure, we will place mice in specialised cages that will allow us to monitor various parameters non-invasively and continuously. This will replace requiring regular handling by/presence of an investigator to make these measurements at regular intervals, which will reduce stress from handling.

Why can’t you use animals that are less sentient?

Mice are the lowest mammalian species for which we have evidence for similarities to humans in terms of systemic energy balance, which is why we chose to use mice for our studies. The energy sensing mechanisms are only fully developed in adulthood as many of

the cell types that are involved in this process would mature after birth. This is developmentally sensible as the nutrient requirements/provision pre- and post-weaning are different. As the effects of obesity and T2D are mainly concerned with effects in mature animals, our studies require the use of adult mice. We cannot conduct our studies on terminally anaesthesised animals as we will be measuring feeding behaviour as an output, and this needs to be conducted on mice that are freely moving.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will adhere to best practice in post-operative care and peri-operative administration of analgesia as advised by the NVS. Frequency of weighing will increase in the 7 days after surgery in order to monitor weight loss and supplement the use of welfare scoring sheets.

Monitoring will also be increased after any substance administration, particularly when substances/doses are administered for the first time. When substances are delivered directly into the brain, animals will be monitored closely for the first 30 minutes, so that we are able to detect any immediate unexpected effects of intra-cranial infusion.

We will acclimatise mice to the new cage environment which will allow us to monitor physiology in a non-invasive manner (e.g. use of the Phenomaster) to limit stress.

After every experiment we critically appraise what we do to seek out any ways to improve our models to refine procedures and endpoints, and reduce harm to animals. We will use welfare scoring sheets to empirically assess whether any modifications are effective.

We will use Cre-strains such that the genetic manipulation will be targeted only to cells that express Cre-recombinase in the animal. This will limit the effects of the genetic manipulation only to the sites that we are interested in probing, minimising off-target effects, and also lowering the animal numbers needed (due to a decrease in experimental noise from off-target effects).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to LASA guidelines (3Rs section) to minimise harms, use the PREPARE guidelines in the planning of our experiments, and publish protocols and data as recommended by the ARRIVE 2.0 guidelines to ensure reproducibility of data.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are kept up to date on 3Rs via periodical emails from our animal unit and from the NC3Rs newsletter, and have incorporated these in to our protocol. We will also monitor advances in the scientific literature that might be adopted to refine our procedures further.

66. In vivo imaging to develop cancer therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

cancer (solid tumours), therapeutics, molecular imaging, cell tracking, immunology

Animal types	Life stages
Mice	adult, pregnant, aged, 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?

We will employ new methods to visualize how cancer develops and spreads over the course of time, and use this new insight to study ways to efficiently treat cancers with better therapies

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Advanced cancer is a global problem that affects millions of people every year. The numbers of people that die of cancer are predicted to increase over the next 20 years unless there are improvements in the early detection and treatment of cancer. Thus there is

an urgent need to improve our understanding of multiple aspects of cancer if we are to increase patient survival rates; these aspects include, for example, how cancers evade detection by the body's own defenses, how cancers continue to grow rapidly despite being treated, and how some cancers spread so swiftly throughout the body.

This research will inform us better about human cancer and how it progresses as well as how to treat it efficiently. It will employ relevant animal models of and provide insights necessary to develop new and improve existing new cancer treatments.

What outputs do you think you will see at the end of this project?

New validated cancer models that can be traced throughout the body by imaging. These include models for breast, liver, lung, ovarian and skin cancers.

An expanded platform for in vivo traceable cell and gene therapies that is compatible with clinical use in humans. This includes relevant preclinical datasets that form the basis for their possible translation into the human setting.

Quantitative imaging data describing cancer progression and spread within the body in different cancer settings.

Quantitative imaging data validating therapies and therapy combinations intended to interfere with cancer progression and spread.

Who or what will benefit from these outputs, and how?

This project will enable us to better understand and quantify cancer development, progression and spread throughout the body by applying advanced imaging techniques. Through application of improved molecular imaging methodologies we will be able to better develop, monitor and validate next-generation cancer treatments.

We will use quantitative measures for cancer formation, growth and spread in non-invasive ways and thereby gain a better understanding of these processes. Our focus on repeat-imaging methodology will permit us to do this with less animals as compared to conventional approaches and also gives us access to statistically more sound data. The improved understanding of cancer that we obtain in this manner will be exploited to develop new and improve existing anti-cancer therapies and, importantly, validate them in a variety of relevant tumour models. Our research contributes to more reliable, more reproducible and also faster development of anti-cancer therapies.

Ultimately, the beneficiaries will be cancer patients who will be presented with better therapeutics and with better ways to monitor these therapies, and the health systems who can draw from better tailored therapeis and avoid debilitating and expensive treatments in patients who will not benefit.

How will you look to maximise the outputs of this work?

We will ensure the experimental design of every experiment is appropriate to answer the research questions we ask, using only relevant disease models, as well as the most refined available methods. We will further ensure that any materials intended for input into animal experiments are properly validated. Moreover, we will ensure that only appropriately trained personnel is involved in experimentation at every level (in vivo but

also ex vivo when using animal tissues). All this is aimed at minimizing failure of animal experimentation and waste of valuable animal-derived tissues.

As for newly developed models and imaging platforms, we will disseminate information as soon as reasonable to enable swift adoption among the local, regional and international research communities. Generally, we will aim to publish all research outcomes in line with the funders' and institution's policies relating to research exploitation and dissemination.

Species and numbers of animals expected to be used

- Mice: 5260

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 the species is crucial to modelling human-derived tumours appropriately. The model species must support the growth of these tumours, must provide distinct levels of biological self-defense (‘immune system’), and be of a size that is compatible with the technical specifications of the imaging techniques (e.g. resolution) required to visualize the cancers in this species. At the same time it is best to use the species with the smallest self-awareness that meets these criteria, which in this case is the mouse.

We will use young adult and adult animals for experiments and will keep them only as long as experimentally necessary. Aged animals will be avoided whenever feasible as they could spontaneously form additional tumours, which may happen in some strains.

Typically, what will be done to an animal used in your project?

Animals will be induced to grow a tumour (either by giving tumour cells to animals or by manipulating animals to form tumours). These tumours will grow and progress to later disease stages either in the absence or the presence of therapeutic interventions while we will use imaging to visualize tumour and/or therapies in these animals. Animals will receive only one type of tumour. Some animals may serve as special control cohorts and may be subjected to the same procedures while not bearing a tumour.

Once experimental goals or predetermined humane endpoints are reached the animals will be humanely killed and tissues used for analysis and production of experimental data to answer scientific questions posed in this project.

An exception are animals maintained for breeding purposes who will not be induced with tumours throughout their lives. Some specially bred animals may be transferred to different licenses, hence not be culled under this authority.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will develop cancer as a consequence of administering cancer cells to them or inducing cells to become cancer cells. The majority of routes by which we give the

animals cancer will cause only mild discomfort. Some methods require surgery while animals are unconscious (due to general anaesthesia). Tumour cell induction may require the administration of specific agents and we will use previously reported and safe doses of these agents.

The development of tumours and their progression as required in this project will be the cause of moderate adverse affects including cancer spread. We will carefully and frequently monitor tumour growth in each individual animal including clinical markers such as, for example, appearance, indications of pain, weight and food intake.

Imaging requires the animals to be still and often also requires contrast agent administration. We have previously optimized anaesthesia and will use safe ranges of contrast agents. Consequently, no adverse events are expected to be caused by imaging.

For therapy we will use previously determined safe amounts whenever possible.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity any animal will reach in any of the protocols is MODERATE. Most procedures are MILD but animals may experience MODERATE severity as a consequence of tumour formation. We estimate 75% of the tumour-bearing animals to experience MODERATE severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We require the use of animals because

Regulatory agencies require animal data to demonstrate safety and efficacy before therapeutics or companion imaging approaches can enter human trials. Most of the agents to be studied have not been used in man before and require animal data before approval for human studies. In many cases, even if the mechanisms are well established, it is unethical to use the tools (inhibitors, cancer cells etc) required to validate the corresponding targeting mechanisms and to understand the biological mechanisms in humans. Consequently, multiorgan species as closely related as justifiable to humans are required.
Certain aspects of tumour biology and cancer progression/metastasis can only be studied in live animals (e.g. interaction of cancer cells with other body cells, spreading of cancer cells to other body locations, consequences of disturbed blood supply, tumour re-growth

after treatment etc.) because there are complex interactions between different body systems, which cannot be replicated in anything other than an intact animal.

Which non-animal alternatives did you consider for use in this project?

Prior to all in vivo work, human and animal cell-based methods as well as tissue-based methods (from already existing tissues) will be used as relative replacements to answer as many research questions as possible and build solid hypotheses to be subsequently tested in vivo. For example, this includes in vitro experiments in cells that are designed to determine target-binding efficiency, agent toxicity, agent stability; or experiments to form solid theories about the mechanisms of cancer spread, the ways the cancer uses to render treatments ineffective, and how the cancer overcomes the body's own defenses. Any new/improved candidate agents, which is found in this manner to be unlikely to succeed in animal or later human trials will be eliminated at this stage.

In very few exceptions it might possible to envisage replacement using humans, namely if an already approved therapeutic or imaging agent is repurposed for application in a different condition or application.

Why were they not suitable?

In vitro experimentation is suitable to reduce the candidate agent field to the most promising ones, and this is what we will employ as a general principle. It is not suitable, however, to model the complex interactions that take place in living multi-organ species. This includes aspects such as pharmacokinetics/dynamics and in vivo tumour progression/spread/heterogeneity as well as the various selection pressures that impact on these disease aspects. As these cannot be studied for the first time in humans for ethical reasons, 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?

A total of max 3100 tumour models and 250 naive animals will be needed to complete the work programme. These animals will be recruited from commercial breeders (or existing on-site breeding under license from commercial breeders; ~75%) as well as from our own colonies (~25%).

For the planned research, we estimate that we will need 2750 animals across different strains to supply us with the necessary not commercially available mice (see above), whereby the total number already takes into account different needed strains and the need to focus on one gender in some studies (i.e. breast cancer models require female animals only for tumour models). We expect ~30% of them to be used for cancer models.

This means ~2510 animals to be sourced commercially plus 2750 home-bred animals (to provide the 840 needed ones for tumour models), totalling 5260 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Imaging to determine tracer distribution rather than conventional ex vivo organ counting is a major contributor to reduction. It allows repeated time-dependant measurements on the same animal as animals are only killed at the last time-point; for example, if a study involves six time-points, the animal numbers are reduced to one sixth. Since each animal serves as its own control, the data are statistically also more robust, which in turn leads to further reduction as smaller cohort sizes are required (because inter-animal variability no longer needs to be taken into account at the experimental design stage).

Moreover, not only contrast agent distribution in vivo, but potential time-dependent and unexpected redistribution can be detected through serial imaging. The same accounts for therapeutics agents that can be tracers, either through chemical modification with an imaging agent, or, in the case of live cell therapies, through tracking these cells by imaging. The detection of cancer spread can through imaging be assessed in a more holistic way, and the reliance on studying pre-set organ sets or sacrificing animals prior to the development of metastases can be dramatically reduced.

All these attributes contribute to a greatly improved benefit:cost ratio (benefit=data quality/quantity, cost=animal numbers/procedures).

Pilot experiments will be performed where necessary on small cohorts, to provide statistical data allowing animal number estimations for definitive larger experiments. Where possible, multimodal imaging will be used to multiplex and study two or more agents/mechanisms simultaneously in the same animal. Multimodal cross-validation will yield superior data because each animal will be the control for itself – data will be intrinsically paired. Sometimes, it will be possible to study >1 tumour per animal, with the same advantages.

A typical study might be planned as follows: For testing of a new molecular therapeutic targeting metastatic spread in mice (Goal 1) including repeat imaging at 4 different time points, we estimate animal numbers using a power calculation (two-tailed t-test, $\alpha=0.05$, power=0.9, typical measurements of 7.8±3.5(signal) and of 2±1(noise) yielding $\delta=5.5$ with $\sigma=3.4$) yielding N=9 animals/group. To account for tumour growth variation and complications with anaesthesia, specific diets, interventions or treatments, we would oversubscribe each cohort by one animal, i.e. plan to investigate 10 animals/group and 20 in total. This approach ensures that accidental unexpected events do not result in the termination/failure of the experiment due to not reaching statistical significance because of sudden insufficient cohort members, and avoids a consequential repeat of the experiment. For the cost of 2 animals it is ensured that unexpected events to not cause the loss of otherwise 18 animals.

[For reference: the comparative study without repeat-imaging would need at least 80 as at every time point one cohort of each control and experimental animals would need culling to assess the effect. Larger cohorts sizes would also be likely as the standard deviations might be larger due to overcoming inter-animal variability when comparing different time points].

If target-to-background (signal-to-noise), or difference between experimental and control groups, is expected to be large (>1 order of magnitude change, e.g. in the case of comparing distant tumour burden in a spontaneously metastasizing tumour model that is

treated with an effective anti-metastatic agent), or found to be so by pilot experiments, statistical significance may be achieved with fewer animals/group. However, we will not use less than 3 animals per group to comply with generally accepted scientific reproducibility criteria in the field.

In summary, imaging in the context of this project, significantly reduces animal use while at the same time providing better and additional data compared to previous methodologies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The experimenters will be advised by the licence holder, deputies, and other members of the preclinical imaging community (which comprises biologists, chemists and technical staff) to ensure that the experiments are scientifically valid and eliminate those that are not.

The experimenters will be thoroughly trained in all necessary procedures to keep 'user-introduced' variability to a minimum. Certain measurements will be preferably taken by the same researcher to ensure consistency; one such example are calliper-measured growth curves of superficial tumours (which tend to be prone to user-to-user variability of using the callipers).

We will operate a regimen of open communication of our research within the our Department/School/Faculty to ensure any possible synergies between the studies of different researchers can be capitalized on (e.g. through "combined controls" and/or re-use of harvested tissues for different scientific purposes).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal models: We will use cancer models established in mice through either (i) injection of cancer cells, (ii) implantation of tumour pieces, or use of transgenic animals that can either be (iii) induced to form tumours ('user-controlled') or, (iv) in limited cases, spontaneously form superficial tumours. We will use non-metastatic and metastatic (inter- and intra-organ) models and models suitable to study tumour heterogeneity, tumour evolution, and treatment resistance. Only the models and progression stage that are key to a required outcome of each project goal will be used.

Methods: The use of tumour and metastasis models is necessary to preclinically validate new or improved cancer-specific therapeutic agents and investigate tumour progression/spread. New and improved agents will be validated in this project and hence, must be used in conjunction with the tumour models. Differing tumour models such as non-metastatic and metastatic ones are necessary to study tumour progression and

metastasis and tumour heterogeneity and evolution; in this context repeated imaging with specific molecular imaging contrast agents is a very powerful method to quantify cancer spread and molecular changes in cancerous tissues over time. Various animal preparation approaches to define and control the tumour microenvironment are essential for these studies; this includes modification of the immune system (either by using genetically altered strains or by sublethal whole-body irradiation or by immunomodulatory agents). Specific insight into molecular processes can be obtained either via genetic approaches (requiring switch on/off of certain genes in genetically altered animals) or via pharmacologic modulation approaches (e.g. inhibitor molecules). Surgical resection of primary superficial tumours is in some cases necessary (some metastatic models) to reach the experimental objective, because too fast primary tumour growth can lead to reaching endpoints before metastasis reaching levels required for obtaining the experimental goals and/or statistically sound data.

To discover what causes treatment resistance in cancer, the use of tumour models together with therapeutic approaches is required, whereby repeated imaging serves as a very powerful method to inform on time-dependent changes in the very same animal. Therapeutics include chemo-, targeted-, and immuno-therapeutics as well as radiotherapy.

Common to all currently available imaging methodology is the requirement of the animals remaining motionless during imaging, which renders general anaesthesia essential. General anaesthesia is also necessary for surgical procedures (e.g. orthotopic tumour implantation in the liver or lung lobe, or intracardiac administration of cancer cells for some metastasis models).

Why can’t you use animals that are less sentient?

Mice are the species of least neurophysiological sensitivity that provide the capability to support the growth of human-derived tumours (xenografts) and present with an immune system sufficiently similar to humans (for syngeneic murine cancer models). Mice also present with the minimum size compatible with the scale of resolution/movement associated with the imaging techniques we plan to use (resolution of the whole-body imaging techniques is of the order of 0.5-1mm).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be humanely killed and their tissues used for downstream analyses once the experimental goals or predetermined humane endpoints are reached. If the experimental goals can be reached earlier through a procedural refinement, e.g. through the use of imaging or through the use of an improved drug formulation, then we will implement this as a refinement.
For example, we have very recently developed an in vivo cancer cell tracking method that reports the tumour mass of living tumour cells in 3D at depth; this enabled us to quantify the primary tumour volumes occupied by living cancer cells much more accurately compared to superficial calliper measurements or luciferase measurements, and it enabled us to quantify tumour burden at distant metastatic sites such as lymph nodes and the lungs. This increase in accuracy permitted earlier termination of experiments as significant differences in distant metastasis and on the primary tumours were detected earlier by this non-invasive imaging technique. This meant that none of the animals in the study reached a humane endpoint and all were sacrificed well before the manifestation of clinical signs in this study.

To minimise any transient pain from injection, we will use inhalation anaesthesia whenever feasible. Furthermore, we will use pain relief after surgical procedures, which are necessary for the establishment of some tumour models.

To minimize distress from handling and/or stemming from procedures normally performed while animals are awake, we will combine these steps with procedures that require anaesthesia; for example, while an animal is under anaesthesia after i.v. administration of a molecular probe and awaiting transfer onto the respective imaging device, we will also perform blood sampling, weighing and superficial tumour measurements if applicable.

We will continue to develop and apply such approaches to reduce severity, transient pain and distress of the animals as well as implement approaches developed elsewhere for this purpose if feasible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the principles laid out by the UKCCCR guidelines and by Workman et al (Brit J Cancer 2010) as well as by LASA, for example, when performing surgical procedures (Guiding Principles for Preparing for and Undertaking Aseptic Surgery) and original literature such as for pain assessment in laboratory animals (Langford et al Nat Meth 2010).

Accurate reporting of experiments is an integral part of reliable and reproducible research, hence we follow the ARRIVE framework (now ARRIVE 2.0: Percy du Sert PLoS Biology 2020).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All team members holding PILs also subscribe to the NC3R newsletter (https://nc3rs.org.uk/subscribe- our-newsletter) and will attend NC3R online education/webinars or in-person workshops if relevant to this project. The team is in regular exchange with named persons and animal unit staff, which is another way of being informed about 3R news. When available and if relevant, local 3R symposia will be attended by the team. Relevant scientific literature published in journals such as LabAnimal (Nature Publishing Group) and JoVE are regularly searched by the team for new methodologies, which are then assessed on whether they offer improvements in light of the 3Rs that can realistically be implemented locally.

67. Skin immune responses in inflammation, infection, and cancer.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Skin-inflammation, Innate-immunity, cancer-immunology, Cutaneous-lymphocytes

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand, at the molecular and cellular level, the role of human skin immune responses in mechanisms of disease, potential treatments, and vaccination. As well as contributing to an understanding of disease pathogenesis, we aim to translate our findings to changes in clinical practice.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Skin frequently represents the first point of contact with bacteria and allergens, yet we still know relatively little of the role of the skin barrier and skin immune system in reacting to such challenges. This is crucially important in understanding the mechanisms of skin

diseases and related diseases, and for optimising approaches to drug and vaccine delivery to this important tissue.

Skin is also one of the main organs that is directly exposed to carcinogenic materials and UV irradiation. Understanding the effect of such onslaught on the skin immunology, and the consequence to development of cancer and skin inflammation can set a rational for developing treatments.

What outputs do you think you will see at the end of this project?

Outputs will include publications describing mechanisms of skin inflammation, pathways of antigen recognition in the skin and draining lymph nodes, and intervention regimen for pathological skin inflammation conditions. Outputs will also include patent applications and candidate compounds with therapeutic application in skin inflammation diseases, as well as treatment of subsets of cancers that are either located in the skin or bare similarities to some of the cells that normally reside in the skin.

Who or what will benefit from these outputs, and how?

Novel findings, delineating pathways in skin inflammation and novel reagents used for modulating these pathways will be published within the 5 year duration of this licence. Patent applications and assessment of products that will be developed for preclinical testing and clinical use may extend beyond the time frame of this licence (beyond 5 years). Novel mouse experimental setups may also benefit research in other barrier tissues such as gut and lung. Such traversing applications will be explored within the time frame of the project (5 years).

Where widely applicable new treatments with public interest will be developed, a wider publication audience will be sought through our funders and local communications advisors.

How will you look to maximise the outputs of this work?

Dissemination of new findings will be initially done in lectures, conferences and subsequently in scientific publication. We will use such occasions to draw on expertise from colleagues and set up collaborations in order to take advantage of the most advance technology that we can access.

Where possible unsuccessful approaches will be embedded in publication and discussed in meetings.

Species and numbers of animals expected to be used

- Mice: 18,500

Predicted harms
Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The use of mice, rather than any other organisms, is based on the balance between similarity with the human immune system versus the availability of transgenic models, the

ability to work with sufficient number of animals to achieve statistical significance, and the large body of published scientific evidence that exists and can be built upon and referenced to.
The diseases that are modelled in mice often differ in some aspects from the equivalent diseases in human (e.g., tumours tend to grow faster in mice. The models of atopic dermatitis is more acute than chronic, as it is in humans). Nevertheless, when examining aspects of the diseases such as response of specific cell types to treatment, there is sufficient similarities to make conclusions that are transferable to human biology and disease.

Typically, what will be done to an animal used in your project?

Two types of models will be used in this licence.

The first involves creating inflammation in the skin that mimics such conditions as Psoriasis, Allergic dermatitis, and Atopic dermatitis. These will be generated by topical application of irritants or allergens or by injection of such compound (and organisms such as bacteria) into the skin (typically the back of the ear or the back of the animal). The symptoms that will develop are inflamed skin, redness, thickening of the skin, flaking skin. Treatments involving novel manipulation of the immune system will then be tested for their ability to ameliorate the symptoms of these diseases. The treatments will be delivered either locally by injection/topical administration, or systemically by injection into the blood, or the peritoneal cavity. Such experiment will typically last two to three weeks and may involve a single injection to induce the disease (or eight daily application of cream to induce the disease) followed by repeated injection (typically two to three times per week) of the treatment. Initial experiments will mainly be conducted on mice with the C57BL/6 genetic background since the progression of the disease in this strain has been consistently shown to mimic several aspects of the human disease. The use of this strain also permits us to make use of extensive range of transgenic and knockout mice that exist on the same background, in order to investigate the effect of specific genes and cell populations that are involved in the development of the skin inflammation symptoms.

The second involves implantation of tumours by injection of tumour cells either in the skin or in the blood. This will be accompanied by treatments that are designed to manipulate the immune system to interact with the tumour and restrict its growth. These experiments will typically extend to three to four weeks while the tumour develops and in cases where growth of the tumour is inhibited by the treatment, experiment will extend further in order to monitor the delay in tumour growth. Treatments may include repeated injection of compounds (typically two to three times per weeks over a period of two to three weeks).

What are the expected impacts and/or adverse effects for the animals during your project?

The main impact on animals will be the local symptoms of the inflammation. These include redness of the skin, thickening of the skin and flaking skin. Small areas may also develop scarification and in some cases first signs of necrosis (this latter will be a humane end point).

In the case of experiment with implanted tumours, the increase in tumour size may reach $1.2cm^3$, however due to the flexible nature of the skin in mice and the location of the implanted tumours on the side and back of the mice, this does not impede their movement or behaviours and does not impact on internal organs. For intradermal implantation where

there is a greater risk of ulceration, the maximum tumour volume is reduced to 0.8cm3 (volume is calculated by measuring three diameters and using the formula: H x D x W x 3.14/6)

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

11% Subthreshold

40% Mild

49% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Using a mouse model of skin inflammation allows analysis that complex interaction between different organs in the animal, including migration of subsets of immune cells from the blood stream, lymphatic system and even bone marrow. Mimicking the symptoms of psoriasis and dermatitis disease involves restructuring of the tissue and formation of features such as flaking skin and eczematous lesions. This process involves several cell types and requires the *in-vivo* tissue architecture in order to develop .

Development of tumours in mice is also a multifactorial process which involves not only the growth of the cancer cells themselves but also a creation of microenvironment around the tumour in response to the tumour growth and molecules that it secretes. The presence of tumour in one location also affects the entire animal body in ways that are impossible to recreate *in-vitro*. Since we are interested in the interaction of immune cells with the tumour, the *in-vivo* setting and interaction with distal parts of the animal are vital in the understanding and development of treatment that can be translated into human therapies.

Which non-animal alternatives did you consider for use in this project?

We are using several *in-vitro* experimental methods to analyse skin tissue from healthy individuals as well as patients with psoriasis, atopic dermatitis and contact dermatitis.

We use imaging analysis linked with single cells analysis of gene and protein expression of those human tissue sample and use this analysis to predict gene and proteins that play a causative role in the development and susceptibility of the skin conditions.

We isolate T cells from peripheral blood in order to identify cells that can interact with skin cells under inflamed condition. These cells are cloned and used for in-vitro analysis of their pro-inflammatory, or anti-inflammatory properties.

We experiment with the use of skin obtained from healthy individuals (from discarded tissue from plastic surgery) to recreate in vitro tissue that can retain function for a limited period.

We use skin tissue derived from patients and healthy donors to isolate several types of skin cells that are involved in development of skin inflammation (e.g., Langerhans cells). We can maintain these cells in a functional state for a limited period during which we can co culture them in vitro with T cells to assess the nature of this intracellular interaction.

We use a model of ex vivo suction blister analysis in which volunteers' skin is blistered and allergens are injected in the blister location. Cellular and fluid infiltrates are then recovered for analysis of the response to those allergens.

Why were they not suitable?

These in vitro and ex-vivo techniques are very useful in generating hypothesis about the causes of skin disease and can form the theoretical basis for developing treatments, but they do not allow multi tissue interaction analysis, and development and testing of therapeutic treatments with multi-factorial analysis of symptoms of the disease.

Other limitations include the frequency and amount of tissue that can be recovered from patients, the limitation of tissue recovery at early stages of the disease, before the symptoms develop, and the limitation on the novel experimental treatment that can be tested.

Reconstructed cellular in-vitro systems also suffer from the bias that we introduce by choosing the cell types that we co-culture.

With respect to cancer model analysis, the current in vitro methods for reconstructing tumours do not fully capture the 3D nature of cancer tissue with all the associated structural and functional microenvironment or the gradual infiltration of different cell populations and blood components.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used in this project is based on the estimated requirements for experiments in each model as well as the number required to breed and maintain a productive colony.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Web application, Experimental design Assistant (https://eda.nc3rs.org.uk/eda/landing), has been used to rationalize optimal numbers of animals for future experiments.

Shared control groups are used in combined experiments where more than a single question is addressed to reduce the need for repeated control groups. (the simplest example being when using three different blocking antibodies in identical experimental setting, there is only a need for a single Isotype control group).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Best practice is followed for colony management to ensure we do not breed animals that are surplus to our experimental needs. The Establishment has a dedicated colony manager who can advise. There is also a mouse database from which we can source data for our strains that assist in colony management

Pilot studies that determine the parameters for group size calculations will be used to optimise the number of animals used in the experiments. Collection of multiple tissues at the end of experiments (occasionally storing them for later analysis) will reduce the need to repeat experiments for the purpose of assessing different aspects of the experimental 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For the analysis of inflammatory skin conditions we will be using several models which differ in the stimulus that triggers the skin disease. These includes a cream containing an inflammatory compound or allergen, or intradermal injection of a bacteria that causes skin disease. In all cases these substances will be localised rather than systemic in order to reduce the overall suffering. Most experiments will be restricted to two to three weeks in order to limit the duration of skin inflammation.

For the analysis of T-cells from Psoriasis patients and dermatitis patients, we will use immunocompromised mice, that do not reject injected human cells. These mice will also be injected with human cell lines, or human tumours or express human proteins, in order to examine the function of the human T-cells with the human cells and proteins. In this model the reaction of the T cells will be assessed by measuring tumour size, or sampling the blood. Typically these experiments will last no more than three to four weeks. The tumours will be implanted subcutaneously at the side of the mouse (near the back) and will not cause the mice any pain or distress due to their location and the fact that they will not

impose on any internal organs. The skin of mice can be quite loose and tumours growth of up to 1.2cm^3 do not cause any pain or distress to the animals. Implanted tumour models will be used in immunocompetent mice in order to test therapeutic compounds. These are well established models which have been shown to cause little to no pain or distress to the animals.

In a few experiments mice will undergo bone marrow transplant which involves irradiation that kills the cells in the mouse's bone marrow, to be replaced by new bone marrow that is injected shortly after the irradiation. This treatment causes some illness but it is short term (about a week) and the mice recover with no lasting harm. This type of experiments is an important tool for assessing the role of the blood cells, and specific proteins expressed in the blood cells, in the progression of the disease.

Why can’t you use animals that are less sentient?

The use of mice rather than other animals, further down the evolutionary development tree, is based on the balance between similarity of the immune system to that of humans, and the availability of transgenic models and the ability to work with sufficient number of animals to obtain statistically significant results.

In particular when working on skin immunology, animals such that are less developed evolutionarily e.g. common models such as zebra fish and drosophila flies, are not suitably close to human skin biology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experimental protocols are formulated to minimise stress or harm to the animals and are based on the observations in pilot experiments, repeated past experiments and published observations.

We continually refine our methods, including changing humane end points, assessing and adding new monitoring techniques (e.g., equipment for measuring tumour size, or ear inflammations), and reducing number of procedures to the minimum necessary for the desired effect (e.g., reducing the number of injection of compound once a sufficient effect has been observed).

We regularly inspect animals to determine their welfare (e.g., take weight measurements and observe changes in their social behaviour and appearance).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The design of experimental procedure will follow best practices guidance described by ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines/inclusion-and-exclusion-criteria), and PREPARE guidelines (https://norecopa.no/prepare)as well published guidelines on tumour models (Workman P et al 2010 British Journal of Cancer (2010) 102, 1555 – 1577.) and skin inflammation models(Lowa et al 2018 Exp. Dermatol.27: 478-483), as well as general practice guidance (e.g https://www.rspca.org.uk/webContent/staticImages/Downloads/WelfareAssessmentProtocolsFull.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly review the literature relating to animal models and improved more refined models that relate to our work. We also discuss ways of improving our models with other groups and with the Named Veterinary Surgeon. We also access specific literature on animal welfare through the nc3Rs web site and regular links sent by the 3Rs Information Officer.

68. Novel Biologicals for Tumour Immunotherapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

immunotherapy, cancer, biologics, antibodies, adjuvants

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to investigate novel biologic medicines suitable for immunotherapeutic treatment of cancer patients with the goal to develop safer and more potent strategies based on the use of immunotherapeutic antibodies, nanobodies and adjuvants.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Immunotherapy of cancer has shown to have great potential in the treatment of cancer patients. However, many of the current immunotherapeutic medicines in routine clinical use for treatment of cancer show a high incidence of adverse events. By modifying existing therapeutic approaches, the safety profile and the potency of immunotherapeutic medicines can be improved. Cancer patients will greatly benefit from more efficient biologic medicines with improved safety profiles.
What outputs do you think you will see at the end of this project?

At the end of the project we will have a better understanding of

how to efficiently deliver biologics such as adjuvants to the tumour tissue using antibody conjugates

what biologics are most effective in promoting anti-tumour immunity when delivered to the tumour tissue

what treatment schedules for specific biologics are most efficient in promoting anti-tumour immunity

Who or what will benefit from these outputs, and how?

In the short term, the project will allow us to develop specific antibody-adjuvant conjugates for targeted delivery to the tumour tissue with optimised potency and safety profiles.

In the long term, the tumour immunotherapy field will benefit from these outputs by recognising that existing therapeutic antibodies that target tumours can become even more effective in cancer treatment by combining them with the immunostimulatory properties of adjuvants in form of antibody-adjuvant conjugates. The project may also identify novel biologic products other than antibody-adjuvant conjugates with great potential in tumour immunotherapy. Again, the tumour immunotherapy field will benefit from these discoveries.

How will you look to maximise the outputs of this work?

The outputs will be published in peer reviewed journals and the work will be presented in international conferences including the communication of unsuccessful approaches. New collaborations will be sought based on the findings in order to work on the translational aspects of the treatment approach.

Species and numbers of animals expected to be used

- Mice: 1600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the ideal model organism for this project because of the availability of a large array of tools to investigate immune responses and tumour growth in these animals. There is a huge arsenal of genetically altered mice, anti-mouse antibodies and mouse-specific accessory kits for the investigation of mouse immune responses available to study the mechanistic details of anti-tumour immunity in a variety of mouse tumour models. The induction and the effector arm of anti-tumour immune responses cannot be explored fully in vitro due to the spatial location of different parts of the immune system and the high number of different immune cells and different immune effector molecules that form these responses in specific spatiotemporal patterns.

Juvenile mice (5-8 weeks of age) are used since engraftment of mice tends to be more efficient in younger mice. However, adult mice can be used and will be used depending on the experimental design and availability of specific mice such as gene modified mice.

Typically, what will be done to an animal used in your project?

The mice will undergo immunotherapeutic interventions. Immunotherapeutically treated mice may be injected with tumour cells or may have developed tumours prior to the therapeutic intervention or tumours may be injected or develop after the intervention. In some experiments, mice may not be injected with tumour cells or develop tumours and in other experiments, mice may be injected with tumours without being treated.

For investigation of cellular mechanisms underlying the observed immune responses, mice may receive adoptively transferred cells or may be depleted of specific cell types. Adoptive transfer of cells may require irradiation of the mice in order to create a niche for the adoptively transferred cells.

For monitoring of immune responses and tumour growth, mice may undergo whole body imaging and blood sampling throughout the experiment. In addition, mice may undergo immune testing in vivo to quantify the scale of specific effector functions induced by the therapeutic intervention.

At the end of an experiment mice will be culled by a schedule 1 killing method or mice may undergo terminal anaesthesia in combination with perfusion or exsanguination.

A typical immunotherapeutic intervention experiment (protocol 1) without optional procedures will last 7 days. The duration of tumour intervention experiments (protocols 2-4) depend on the tumour model that is used and can last from 18 days (e.g. B16 pseudo-metastasis model) to 28 days (e.g. humanized mouse model with HER2-expressing tumour cells) or can last several months (e.g. genetic tumour models).

Similarly, the number of procedures can vary from 1 (e.g. most simple immunotherapeutic intervention experiment or protocol 4 without whole-body imaging) to 20 in typical experiments involving several steps (e.g. humanised mouse tumour model with immunotherapeutic intervention and blood sampling and whole-body imaging). However, the number of procedures may exceed 20 for specific experiments.

Different strains of genetically altered mice may be used under this licence depending on the scientific question that will be addressed. In general, we may use

animals with a genetic modification that leads to the development of tumours in the mice

animals with defects in the immune systems e.g. lack of pattern recognition receptors, lack of specific immune cell types, lack of specific cytokines or cytokine receptors

animals which are transgenic for specific antigens or which express specific reporter molecules that can be used for investigating immune responses
animals which are transgenic for specific human markers / receptors that can be targeted with immunotherapeutic molecules.

What are the expected impacts and/or adverse effects for the animals during your project?

Possible unwanted effects of experiments involving the growth of tumours in mice include weight loss, weight gain, tumours impeding locomotion and tumour ulceration. Furthermore, mice with tumours may show signs of general ill health such as inappetence, piloerection and hunched posture, inactivity or diarrhoea. However, the likelihood of any of these adverse effects to occur is very low since animals will be culled when tumours reach a specific size and before tumour burden affects the general well-being of the mice. Any adverse effects of tumour growth are very unlikely to be transient and are expected to become more severe over time when the tumour growth further.

In some cases, adoptive transfer of xenografts such as human peripheral blood mononuclear cells (PBMC) can lead to graft-versus-host disease in mice. The likelihood of mice developing graft-versus- host disease is low since animals will be culled at specific time points before graft-versus-host disease is known to develop. However, should graft-versus-host disease manifest in mice, the adverse effects are not transient and will become more severe over time if mice are not killed.

Immunodeficient mouse strains may be used which are more susceptible to infections. However, immunodeficient mice are kept in a bio-exclusive environment such as individually ventilated caging or isolators and rarely experience infections. Should infection occur immunodeficient mice are unlikely to clear the infection irrespective ofthe pathogen.

Expected severity categories and the 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 majority of animals (80%), the level of severity will not exceed mild levels due to the timepoints at which the experiments will be concluded (humane endpoints). From experience, approximately 20% of animals under this licence will reach moderate severity levels with the rest of animals experiencing mild severity levels.

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 interplay between tumours and the immune system and the mechanisms of immunotherapeutic intervention strategies can only be investigated fully in vivo since anti-tumour immune responses involve a high number of different immune cell types and tissue cells and follow specific spatiotemporal patterns which cannot be fully reconstructed in vitro.

Which non-animal alternatives did you consider for use in this project?

Some limited aspects of immune activation can be investigated in vitro such as for

example the activation of innate immune cells by adjuvants. Whenever possible, we will examine specific aspects of the therapeutics in vitro by performing in vitro activation and in vitro binding assays prior to the in vivo experiments.

There are no other suitable non-animal alternatives for use in this project since investigation of the functionality, potency and safety of biologics for tumour immunotherapy requires to understand the interplay of the immune system and the tumour tissues including intact tissue architecture such as lymphatic vessels, lymph nodes and blood vessels.

Why were they not suitable?

The induction of antigen-specific anti-tumour immunity requires the priming of T cells and B cells in lymph nodes and the induction of anti-tumour immunity requires the migration of effector cells to the tumour tissue where the immunosuppressive tumour microenvironment may negatively affect the functionality of the effector cells. The specific connection between tissue or tumour tissue and secondary lymphoid tissue such as lymph nodes cannot be reconstituted in in vitro assays.

Also, the pharmacokinetics and pharmacodynamics of immunotherapeutic drugs such as antibody- adjuvant conjugates cannot be investigated in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We use 30 animals per experiment since this is the maximum number that can be processed experimentally within the laboratory for logistic reasons. We further estimate to perform one experiment per month on average bringing the estimated number of mice to 360 per year and to 1600 mice over the lifetime of the licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We sought internal advice from our statistician colleagues to calculate the required group size based on estimated variabilities within groups in combination with estimated differences between groups. The used estimates stem from experience with similar experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We perform pilot experiments whenever necessary as for example when working with a new tumour cell line or a new immunotherapeutic biologic medicine. The pilot experiments are designed to allow us to refine specific aspects of the procedure (e.g. dose of injected tumour cells, dose and treatment regimen of the immunotherapeutic drug, end point of experiment) in order to optimise the experiments. From these pilot experiment we can

calculate how many mice will be required to produce statistically significant results. This largely depends on the variability in the response within treatment groups and the differences observed between treatment groups.

We also work with whole body imaging of bioluminescent tumour cell lines whenever possible in order to maximise the data obtained from the tumour models. However, it depends on the tumour cell line / tumour model whether or not whole-body imaging allows us to make the group sizes smaller.

We analyse different read-outs from the same experiment such as serum levels of cytokines and/or antibody titres, tumour growth profiles, induction of antigen-specific T cells, cytotoxic effector functions and immune cell profiling of blood, splenocytes and tumour tissue. Thereby, we gain in-depth insight for various aspects of the immune response from the same experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using exclusively mouse models during this project. Regarding the tumour models, the project includes syngeneic mouse tumour models and also xenograft tumour models with human tumour cells. In some experiments, we may also adoptively transfer human immune cells. The project may also include the use of genetically altered mouse strains such as for example NSG mice which allow for effective engraftment with human xenograft tumour cells and human immune cells. In addition, other genetically modified mouse strains may be used such as specific knock-out or reporter mice in order to study specific mechanistic aspects.

The mice will be injected with cells and/or immunotherapeutic drugs and may undergo blood sampling, irradiation, immune cell depletion and/or whole-body imaging in specific experiments.

Mice may be irradiated before adoptive transfer of cells to create a niche for the transferred cells and enable efficient engraftment. From experience, we know that sublethal doses of irradiation are sufficient for most of our experiments (e.g. 3.5Gy). At this dose, mice do not show any adverse effects of irradiation. However, the dose of irradiation required for successful engraftment varies between mouse strains and in some rare cases we may have to use the maximum dose of 11Gy. For identification of the most suitable irradiation dose for specific mouse strains the literature will be consulted.

Anaesthesia of mice will be performed with isoflurane whenever possible. From our experience, mice undergoing anaesthesia using isoflurane do not experience any adverse effects. Only on rare occasions when usage of isoflurane is not advisable will injectable anaesthetics be used.

The models and methods were chosen to enable the investigation of tumour immunotherapeutic intervention strategies. For understanding the potency and safety of tumour immunotherapeutic intervention strategies, the model has to include an intact immune system and a tumour in addition to suitable tools such as antibodies, recombinant factors and/or gene-modifications that enable the mechanistic investigation of the model. The mouse model is the most suitable for these investigations. The identification of appropriate humane endpoints will enable the investigation of the efficacy of the immunotherapeutic intervention and its underlying mechanisms while at the time ensuring that the harm to the animals will be kept to a minimum.

Why can’t you use animals that are less sentient?

Animal models for studying anti-tumour immunity induction require animals with an adaptive immune system similar to humans. The immune system of less sentient animals such as fish or invertebrates are too different from the human immune system to provide meaningful insight into immunotherapeutic intervention strategies. Also, mouse models have the great advantage that a wide arsenal of tools such as antibodies and genetically modified mouse strains have been developed to study immune responses in mice. Furthermore, most mouse tumour models are well characterised and thereby enable their use for studying a variety of intervention strategies with minimal need for optimisation of the protocols.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our biological service division (BSD) has a very good track record in minimising harms to animals. Refinement of procedures implemented during the previous PPL include:

single use needles - using a new needle for every animal that is injected

increased observation frequency after specific procedures (e.g. surgery, irradiation and adoptive transfer, injection of immunostimulatory reagents)

supportive husbandry measures e.g. mash diet and additional warmth when anaesthesia is used or upon irradiation

antibiotic in drinking water pre and post irradiation

non-aversive animal handling techniques

various enrichments in the cages

The scientists, the NVS and the BSD staff working on a project have pre- and post-meetings for experiments. During these meetings possible refinements are discussed and jointly agreed.

In addition, experimental endpoints will be adjusted to deliver the minimal average tumour size that allows to achieve the scientific objective and the minimum irradiation dose that allows for good engraftment. Also, endpoints will be chosen to avoid mice showing signs of graft versus host Disease (GvHD).

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

In addition, for planning of the research and the experimental procedures on the animals we will follow PREPARE guidelines (Smith et al., 2018. Laboratory Animals 52(2): 135-141; https://norecopa.no/PREPARE). For experimental design, reporting and publication of study data we will follow ARRIVE guidelines (https://arriveguidelines.org/).
Regarding the tumour models, we will follow the Guidelines for the Welfare and Use of Animals in Cancer Research (Workman et al., 2010 British Journal of Cancer 102: 1555-1577).

Maximum blood withdrawal volumes and dose volumes for specific routes of administration will be adhered to as listed in the action plan. In addition, expert input from NVS and NACWO will be thought for new treatment regimens.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Scientists working on animal experiments receive regular e-mail updates relating to the 3Rs by the Named Information Officer. In addition, scientists, the Named Veterinary Surgeon (NVS) and the staff members performing the experiments in the biological service division (BSD) hold pre- and post- meetings for experiments. During these meetings all aspects of the experiments including the 3Rs are considered and discussed and are documented in the meeting notes. Refinements are jointly agreed on and implemented for future experiments following the same procedures.

69. Preclinical models of haematological malignancies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

blood cancer, targeted therapy, xenograft, preclinical models

Animal types	Life stages
Mice	pregnant, adult, 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?

The overall aim of the project is to accelerate the entry of new and effective treatments against haematological malignancies to the clinic. For this we aim to develop and characterise novel pre- clinical models of haematological malignancies, in particular novel patient-derived xenografts, and to use these models to assess the efficacy of novel treatment regimens.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Haematological malignancies are the fourth most prevalent cancers in the UK, affecting about 40,000 new patients of all ages each year. While chemotherapy has greatly

improved survival in some malignancies such as for example childhood acute lymphoblastic leukaemia, this can be at the cost of huge toxicities such as infertility, immunosuppression, secondary malignancies or cardiac and neurological problems. For others, such as diffuse large B-cell lymphoma, T-cell lymphoma or acute myeloid leukaemia, the overall survival can be as low as few months with most patients failing conventional therapies and has not improved much over the last decades. There is then a clear unmet medical need for patients with blood cancers. There are many reasons for the persistent lack of efficacious therapies such as poor understanding of the biology of the tumour cells, poor understanding of the role of the microenvironment, the broad heterogeneity/variability within one disease, the lack of preclinical models, including access to patient primary samples and the poor viability of primary cells ex vivo precluding assays.

What outputs do you think you will see at the end of this project?

At the end of this project, we aim to have developed forty novel models across the spectrum of haematological diseases. We will focus more particularly on diseases for which preclinical models including cell lines are rare. These models will be characterised in terms of genomics, growth and drug vulnerabilities. We will also try to develop novel cell lines from this xenograft models, which will considerably facilitate drug screening, the study of mechanisms of tumourgenesis and the mechanisms of drug vulnerabilities and resistance. We are aiming to have tested five different drugs ex vivo (explants or suspension cells) and/or in vivo and that the results will support and inform novel clinical trials. Results from this project will be shared to the scientific community as publications (following the ARRIVE guidelines) and/or conferences. All models will be made available.

Who or what will benefit from these outputs, and how?

In the short- and medium-term, the development of novel pre-clinical models will benefit the academia and pharmaceutical communities. In this project, we will particularly focus on diseases for which the development of novel therapeutic agents has been lagging because of a lack of available preclinical models (including cell lines) and a poor understanding of the tumour biology. The developed models will be made available to use as collaborative work to better understand the pathogenesis of haematological malignancies and to develop better and targeted novel therapies.

In the long-term we hope these models will benefit the patient community. Together with data collected from other studies and by using these models, our understanding of the diseases will be improved and we hope the use of these models will have informed the approval of novel therapeutic agents in clinical trials or as novel standard of care, and lead to novel and better therapeutic options available to our patients.

How will you look to maximise the outputs of this work?

We will look to maximise outputs by advertising the existence of our models, our know-hows and the results of in vitro and in vivo experimental work to potential collaborators both in academia and in the pharmaceutical industry. This will include publications in peer-reviewed journals, communications at conferences and as confidential information to our collaborators. Unsuccessful approaches or negative results will be equally shared.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For this project we will mainly use immunodeficient adult mice. Our main model will be the Nod Scid Gamma (NSG) (NOD.Cg-PrkdcSCID Il2rgtm1Wjl/SzJ) immunodeficientmice which have been shown to be the best model for xenograft studies in haematological cancers but we might use NSG variants when appropriate, or the Nod Rag Gamma (NRG) (NOD-Rag1null IL2rgnull) immunodeficient mice model.

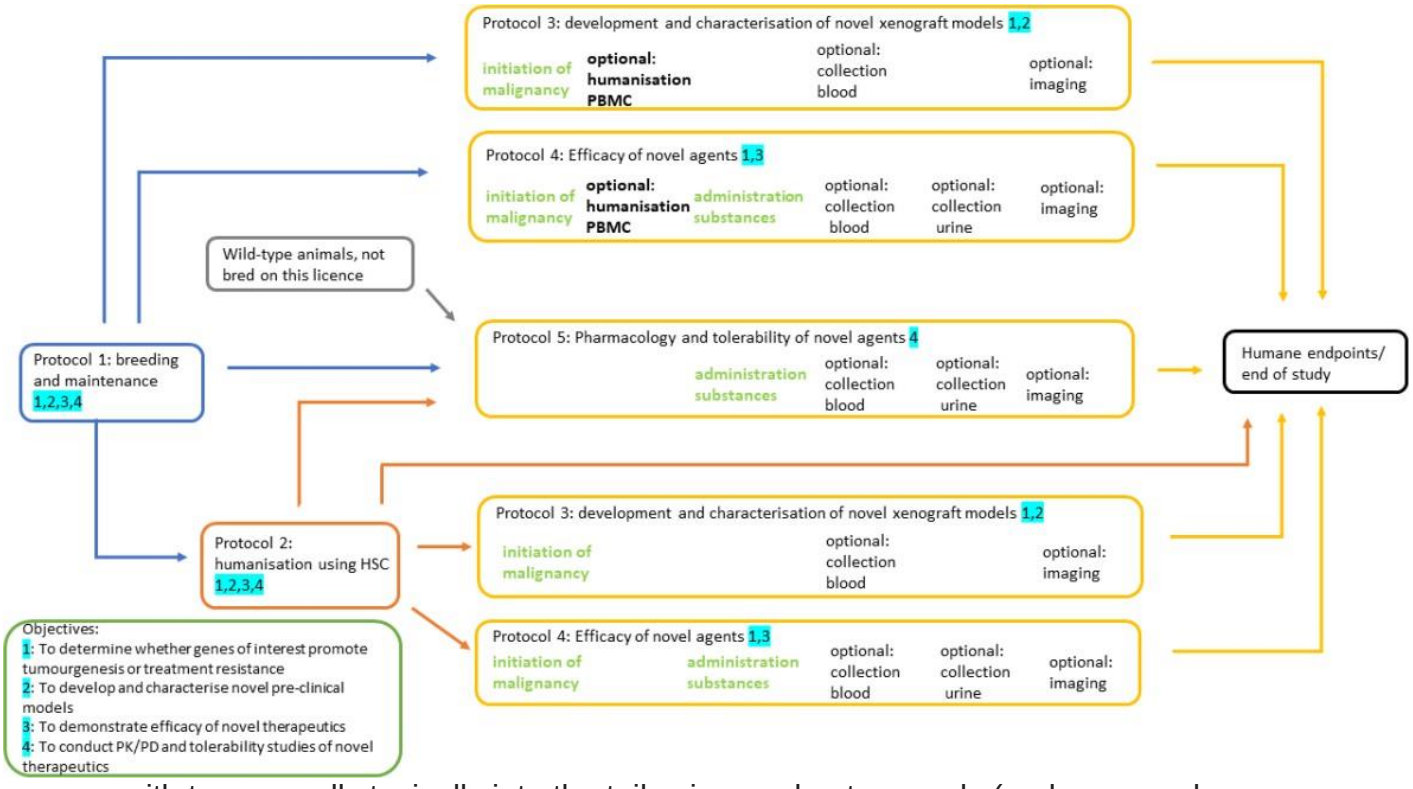
To establish humanised immune system mice, juvenile (5-8 weeks old) NSG, NSG variants or NRG mice will be irradiated to deplete the bone marrow from mouse haematopoietic stem cells (HSC) and to allow engraftment by human HSC after intravenous injection. As an alternative approach we might inject mature immune cells isolated from peripheral blood into adult mice.

Typically, what will be done to an animal used in your project?

A summary of a typical journey of an animal is shown in the figure below:

Throughout this project, we will use primary tumour cells from patients or established cell lines. These cells might be engineered/modified to remove or mutate genes of interest and we will compare their capability to develop tumours or to respond to treatment.

For the development of novel xenograft models, immunodeficient animals will be injected



once with tumour cells typically into the tail vein or subcutaneously (under general anaesthesia), but other routes could be used such as into the intraperitoneal space, into the femur or into the subrenal capsules (both under general anaesthesia). We will only use

these latter routes for the most interesting samples and if engraftment by intravenous or subcutaneous injections has failed. Tumours will be let to grow until they reach the defined humane endpoints (size of subcutaneous tumours, clinical signs, body condition scoring, behaviour, pain). The animals will then be humanely killed, and tumour samples processed for analysis in the lab or reinjected into mice. If six months after injection, animals do not show evidence of tumour growth, they will be humanely killed. Blood samples might be taken to assess the number of circulating human tumour cells. Imaging might be used to obtain further data on tumour growth. If immunotherapy will be evaluated in subsequent pre-clinical trials using these models, we will need to humanise the

mouse immune system and to characterise these humanised models before their potential use. For this, the animals will be injected once, either with human haematopoietic stem cells (after irradiation and before tumour engraftment) or with mature immune cells (no irradiation, after tumour engraftment).

For the assessment of novel therapeutics, established and characterised xenograft models will be used in pre-clinical trials using novel therapeutic agents and we will compare the efficacy of these new agents (or combination) at reducing tumour growth compared to standard of care regimen. These novel drugs will be administered using routes that will reflect the minimum possible harm and distress for the animal in line with the proposed route of administration in patients. We will choose the less invasive route whenever possible and will limit the number of daily administration and the duration of the treatment in line with published guidelines. We might collect blood samples to assess pharmacokinetics (PK) and pharmacodynamics (PD) data in tumour-bearing animals. We might use imaging to limit the number of animals used in these trials. If immunotherapy are evaluated in pre- clinical trials, we will need to humanise the mouse immune system. For this, the animals will be injected once, either with human haematopoietic stem cells (after irradiation and before tumour engraftment) or with mature immune cells (no irradiation, after tumour engraftment).

We expect to have PK, PD and tolerability data for most novel agents we will use in this project. However, if data are not available, we will run small pilot study to evaluate tolerability, and carry out some small studies to determine PK/PD data in immunodeficient animals (without tumour) or in wild- type animals. This will require administration of substances and collection of blood and urine. The routes and frequency of administration will be informed by in vitro and in silico studies. We will choose the less invasive route whenever possible and will limit the number of daily administration and the duration of the treatment in line with published guidelines.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals produced under this project will develop human-like blood cancers, will receive medicines that aim to improve the treatment of disease and reduce the unpleasant side effects of current cancer treatments (chemo and radiotherapy). We expect that the severity will be moderate as animals are likely to suffer moderate impairment of their well-being. This will include the development of tumours and adverse effects linked to the administration of substances and collection of blood samples. A small number of animals might undergo surgery under general anaesthesia as part of the procedures.
Adverse events will be controlled by analgesic when necessary. Any animals that show significant impairment of normal behaviour or signs of distress will be humanely killed.

Humanisation of immune system using human haematopoietic stem cells: Animals will be sub- lethally irradiated to eliminate the mouse haematopoietic stem cells from the bone marrow and to allow engraftment with human stem cells (injection once into the tail vein). Some animals might lose weight about a week after irradiation, this weight loss might reach 15% of the initial weight. We will provide support to the animals such as soggy diet or hydrogel and most animals should recover their initial weight within 3 weeks after irradiation. Some animals might not fully recover their initial weight but their body condition and general behaviour should remain stable.

Humanisation of immune system using human mature immune cells: An alternative approach to humanise the mouse immune system is to inject, once by IV, peripheral blood mononuclear cells (PBMCs) after the initiation of malignancy. The major risk of this approach is the development of xenogeneic Graft vs Host Disease (xGVHD) four to five weeks after injection in NSG strain due to the recognition of the mouse cells by the human T cells. xGVHD is characterised by sudden weight loss, skin fibrosis and hair loss, loss of mobility and increased breathing. We will limit the adverse effects by 1) terminating the study before or on the onset of clinical signs in NSG, 2) using strains that are resistant to xGVHD (NSG lacking MHC system or with humanised HLA system).

Initiation of malignancy will be performed by injection, once, with human tumour cells, typically subcutaneously or intravenously, but for a small number of animals, injection might be intraperitoneally, intrafemorally (under general anaesthesia) or into the subrenal capsule (under general anaesthesia).
The health of animals will be routinely monitored for the apparition of clinical signs such as tumour growth, weight loss, change in appearance, body condition, behaviour, signs of pain, enlargement of internal tissues to follow the engraftment of tumour cells. Health monitoring will be increased as necessary to limit pain and suffering within the moderate severity band.

Administration of substances will be done using routes and frequency that will reflect the minimum possible harm and distress for the animal in line with the proposed route of administration in patients. Maximum volumes for dosing per animal will be ascertained using published guidelines. We will minimise the number of injections when possible. Typically, most animals who will receive drugs by oral gavage, IP or SC, alone or in combination, will receive one or two administrations per day for a maximum of three weeks. However, a small number of animals might require three administrations per day, this will be over a maximum period of one week for IP or SC and over a maximum period of 4 weeks for oral gavage. For intravenous injections, we will limit injections to once per week, with a maximum of 8 injections. Tails will be regularly checked for bruising or infections. To limit the side effects of repetitive injections, we will use the minimum size of needles, rotate sites of injection, assess for bruising or tenderness of the injection areas, allow period of rest if animals show clinical signs (weight loss, lethargy) or signs of pain using the NC3Rs grimace score. For oral gavage, the administered volume will be kept as a minimum to avoid filling up the stomach and weight will be monitored to ensure animals get enough food. Analgesics such as paracetamol might be given to alleviate pain. Whenever possible we will use alternative ways of administration and this could include the implantation of slow release pellet or osmotic pump such as the Alzet minipumps.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals are expected to fall within the mild (40%) or moderate (60%) severity bands.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice offer multiple advantages to help human patient cells grow. Patient cells needs a microenvironment support that has not been fully characterised and cannot be recapitulated in vitro or in tissue culture conditions. We need to use mice to provide the best microenvironment within the different tissues usually affected by blood cancers, ie blood, bone marrow, spleen or lymph nodes for orthotopic models, or to provide blood supply and other support for subcutaneous models.

Which non-animal alternatives did you consider for use in this project?

We routinely use established cell lines and primary patient samples in our investigation. We are holding one of the largest cell line banks in the UK for haematological malignancies and thanks to our close link to the local hospital we have been collecting and using primary samples from our tissue bank for the last twenty years. We are collaborating with many centres in the UK and in Europe to source samples, share best practice and novel technologies. We use a large array of in vitro/in cells approaches, including knockdown/knockout technologies, next-generation sequencing, single cell analysis. We also have access to large sequencing datasets that we interrogate to predict responses to treatment for example and we have initiated collaboration to predict mechanism of resistance using computational systems biology.

Why were they not suitable?

Each model has its advantages and its own limitations. Cell lines are easy to grow, relatively well characterised (even if misidentification and contamination are a recurrent problem), of unlimited supply for diverse mechanistic studies. Cell lines have been an instrumental tool in our understanding of various cancers and the development of novel targeted therapies. However, cell lines numbers are limiting and the scientific community still requires novel cell lines. Some malignancies such as DLBCL for example can be modelled with a bit less than one hundred cell lines, and while this is a great number to work with, this is barely enough to model the heterogeneity and the diversity of the disease seen in patients. Moreover most cell lines have been established decades ago and they don’t represent the tumour cells emerging from patients with resistant/refractory disease to the current therapies. Some malignancies just don’t have enough bona fide model cell lines for researchers to make any significant progress. For example, T-PLL can be modelled by only one cell line, undermining any mechanistic or drug screening studies.

Despite the overall need for cell lines, the cancer community is well too aware of the biological limitations of these models. Cell lines don't fully recapitulate the patient's disease as they have been adapted to grow as a 2D culture on a plastic dish and completely lack the effects of the tumour microenvironment and this could explain in part the high attrition rate in drug development.

Besides cell lines, we use primary patient samples in our studies. While we have a large collection of samples through our Tissue Bank, supply of cells is limited with what we have stored or what we can have access to, with samples that can be limited to few biopsy cores. The low number and low viability of primary cells once put in culture is a major obstacle to any mechanistic or drug screening studies. We have tried to develop patient-derived explants when we had access to lymph nodes or spleen samples but these organs don't seem to be suitable for this type of study as they are of poor viability or too fragile, possibly due to the way they are excised from the patients (blood vessels are first clamped). Moreover, application of drugs is done only by soaking in the pieces of tissue as blood supply is abolished and might limit drug accessibility. We are also trying to develop patient-derived organoids but this approach has its own challenges.

Information gathered from sequencing or multi-omics analysis together with computational predictions are a compelling approach to draw hypothesis but there is still a need to validate those using appropriate 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?

Breeding: We plan to use about 1000 animals for breeding purposes. We endeavour to use all animals (males and females) issued from the breeding for procedural work.

Development of models: we anticipate injecting 80 different patient samples. From our previous experience, about half of these samples are expected to produce successful xenografts. At the moment we are not able to predict which samples will be successful or not. We thus expect 40 patient samples will only be injected as passage 1 (typically 6 animals) and 40 will be injected for 3 passages, about 20 animals in total for each model. In total we estimate we will use 1000 animals for this part of the work.

Assessment of novel treatment regimens: we anticipate we will carry out about 20 preclinical trials over the 5 years of the licence. A typical preclinical trial will consist of 4 treatment arms (Arm 1: Vehicle, Arm 2: current standard of care, Arms 3 and 4: 2 doses of drug of interest) with 5-10 animals per arm. So in total we anticipate we will use about 400-800 animals

Pharmacology study of novel agents: We expect a maximum of 5 novel agents to be assessed for PK/PD studies with a maximum number of 100 animals to be used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For the assessment of novel treatment regimens and the pharmacology studies of novel agents, number of animals will be determined by using the NC3R Experimental Design Assistant and other power calculation tools such as the 3rs-reduction tool (3rs-reduction.co.uk/html/6____power_and_sample_size.html). All novel agents will be first assessed in cells and only the most promising compounds will be evaluated in xenograft models. This pre-assessment in cells will allow us to determine the expected variability of response (noise) and the expected size of the effect (signal).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The animals will be held in a high health facility minimizing bias from the pathogen status of the animals. We will breed animals depending on our study schedules. We will use both males and females.

We expect that for most compounds we will have access to PK/PD data. In case of novel agents never tested before in animals and for which PK/PD data are not available, we will work with our collaborators from the pharmaceutical companies providing the agents to determine target occupancy and drug-target residence time using biochemical studies, studies in cells and computing modelling. PK/PD studies will be complemented with computational predictions of target engagement to select concentration doses for the studies. Moreover, we will run small pilot studies to detect unexpected toxicity. When possible, we will reduce the number of animals in the non-treated/vehicle arm.

Downstream analysis will be optimised to reduce the amount of tissues required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use adult (8-20 weeks) immunodeficient mice for the xenograft studies. Our main model will be the Nod Scid Gamma (NSG) (NOD.Cg-PrkdcSCID Il2rgtm1Wjl/SzJ) immunodeficient mice which have been shown to be the best model for xenograft studies in haematological cancers but we might use NSG variants when appropriate, or the Nod Rag Gamma (NRG) (NOD-Rag1null IL2rgnull) immunodeficient mice model.

We might generate animals with an humanized immune system by transplanting human CD34+ haematopoietic stem cells (HSC) after irradiation or by injecting peripheral blood mononuclear cells (PBMC) to assess novel immunotherapies. In that case juvenile animals might be used. NSG animals are relatively sensitive to irradiation but adequate support will

be provided when needed. NRG animals are more resistant to irradiation and would be used instead.

Whenever possible, the injection volumes and the size of the needles will be as small as possible.

Sampling will require only small amount of blood (typically 50 microliters) as the downstream in vitro measurement experiments are highly effective.

We have developed a comprehensive distress scoring sheet and health check protocols, which allow us to identify any early signs of disease progression and to control pain, suffering, distress and lasting harm appropriately.

Routes of administration for the therapeutic agents will reflect the minimum possible harm and distress for the animal in line with the proposed route of administration in patients. We will follow the LASA guidelines regarding the routes and frequencies routinely. We will consider using subcutaneous mini pump whenever possible.

Why can’t you use animals that are less sentient?

Recent studies have shown that zebrafish could be used as host for xenotransplantation of human tumour cells, with tumour growth similar to what is observed in mice. These zPDXs have also been used in compound screening. There are some advantages of considering zPDX as an alternative of mouse PDXs, as they are less sentient, easier to maintain, allow high-throughput screening and more cost-effective. However, the current limitations of using zPDX are the need of fluorescent-labelled cells, the need of specialized equipment for imaging and injections. Moreover, most studies have used established cell lines as their source of xenograft and up to now very few studies have used patient cells to establish zPDXs. Moreover, this is a relatively new area of research, which will require further evaluation within the scientific community before being implemented and used widely and convincingly. However, zPDX will be looked into as a possible alternative model and if deemed appropriate an amendment will be made to this ppl in the future to include zebrafish and lower the number of mice used accordingly.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our experimental animals have a daily check and are examined at least once weekly. This examination will include recording of body weight, body condition scoring, behaviour, signs of pain, changes to respiration and mobility, evidence of enlarged internal tissues and examination for visible evidence of tumours with size of tumours recorded if present. A distress scoring sheet has been developed based on our previous experience of leukaemia/lymphoma models. Health monitoring frequency will be increased for animals exhibiting subdued behaviour and on the apparition of adverse events. Communication between PPL holder, PIL holders, NACWO and facility staff is paramount. Animals are trained to be tunnel handled during the health checks minimising stress.

Administration of substances and blood sampling:

Other than in terminally anaesthetised animals, dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. Maximum volumes for dosing or blood sampling per animal will be ascertained using published guidelines (The

LASA Good Practice Guidelines Administration of Substances (1998) The LASA Good Practice Guidelines Blood sampling (1998), Turner et al., Journal of the American Association for Laboratory Animal Science, 2011, Vol50, No5, pp600-613, Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider; Members of the Joint Working Group on Refinement, Laboratory Animals Ltd. Laboratory Animals (2001) 35, 1-41, Refining procedures for the administration of substances).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Maximum volumes for dosing and blood collection per animal will be ascertained using published guidelines (The LASA Good Practice Guidelines Administration of Substances (1998), The LASA Good Practice Guidelines Blood sampling (1998), Turner et al., Journal of the American Association for Laboratory Animal Science, 2011, Vol50, No5, pp600-613, Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider; Members of the Joint Working Group on Refinement, Laboratory Animals Ltd. Laboratory Animals (2001) 35, 1-41, Refining procedures for the administration of substances).

We will follow the NCRI guidelines for the welfare and use of animals in cancer research (Workman et al, 2010, British Journal of Cancer). If those guidelines are updated within the lifetime of this licence, the updated guidelines will then be used.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have several routes to stay informed about advances in the 3Rs. At our Institution we have a 3R working group which evaluates the procedures and informs us on advances/experience/guidance to improve our compliance to the 3R. For example, we have quickly implemented tunnel handling of mice when this was highlighted as best practice. Users at our facility meet every six weeks and are encouraged to share their experiences. The regional NC3Rs programme manager is a member of our AWERB committee and attend our User Group meetings. We work closely with our Institution’s NACWO and NVS to discuss ways to improve the welfare of the animals, from housing to procedural work. We also received newsletters from our animal facility that highlight webinars, conferences, articles of interest for our perusal. I am also registered to various animal-focused companies (Charles River, Jackson Labs, Crown Biosciences) and organisations (NC3Rs) as they provide webinars and other useful resources.

70. Tumour-bone interactions in skeletal malignancy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

multiple myeloma, prostate cancer bone metastasis, bone microenvironment, metabolism, aging

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to determine how tumour-bone interactions drive tumour growth and bone disease, and therefore how we can disrupt these interactions to prevent or treat skeletal malignancies. The project is focused upon metabolic, aging and obesity-related changes within the tumour-bone microenvironment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Once solid tumours such as prostate cancer bone metastases, or blood cancers such as multiple myeloma, develop within bone they are largely resistant to current treatments and the malignancies are ultimately fatal. A better understanding how and why tumours like to grow within bone is needed, in order to find new ways to combat disease and new ways to identify those patients at greatest risk.

What outputs do you think you will see at the end of this project?

The primary outputs from the project will be new information, which may lead to new drugs, therapeutic approaches or disease markers. This will typically be communicated by scientific publication or presentation (but also engagement with non-specialist audiences).

Who or what will benefit from these outputs, and how?

In the short-term, those that will benefit from these outputs include scientists interested in tumour and/or bone biology and clinicians, in particular oncologists and bone biologists. They will benefit by an increased understanding of how tumours grow and survive within bone, and the mechanisms that drive this.

In the medium-term, we anticipate that scientists and the pharmaceutical industry will benefit, as our mechanistic findings progress to investigating new therapeutic approaches.

In the long-term, we anticipate that our findings will be translated to the clinic, in the form of new therapeutic approaches or markers of disease progression. Therefore, those that will benefit from this include patients with multiple myeloma or prostate cancer.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work by;

Collaboration with scientists and international societies worldwide to share our findings as quickly as possible

Dissemination of our findings by open access publication in scientific journals, use of preprint servers such as BioRiv and presentation at national and international conferences

Any unsuccessful approaches will be published

Any refinements or good practices identified will be shared via local NC3Rs networks

Species and numbers of animals expected to be used

- Mice: 4400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice because they are the most characterized model for tumour response to therapy and have the widest scope for genetic manipulations. The models we use are the models that most closely replicate human disease, with tumour growth within bone and development of an associated bone disease.

We use young and adult mice, as they provide reliable and well-characterised models of tumour- induced bone disease. We will also use aging mice to study how aging contributes to tumour development.

Typically, what will be done to an animal used in your project?

Mice will be injected with tumour cells and tumours will grow within bone. To study the specific role of the bone, it may also be necessary to inoculate mice with tumours at non-bone sites such as under the skin (in separate studies). Mice may be treated with drugs designed to stop tumour growth, or may be placed on a specific diet designed to increase or reduce obesity. Some experiments may be performed in aging mice. During the course of an experiment, blood will be sampled, and mice may be imaged and have bone marrow samples taken. For most models, the typical duration of an experiment is four weeks, with some tumour models requiring 12 weeks for tumour growth within bone.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects are mainly related to the tumour growth, and may include weight loss, pain, temporary lameness/limping or hindlimb weakness. The project is designed so that such adverse effects have an estimated duration of less that 48h.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold; 40%

Mild; 19%

Moderate; 41%

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 reason that tumour growth within bone and the associated bone disease is so rapid and aggressive is due to the multiple interactions between tumour cells and host cells within the bone that promote both tumour growth and survival and bone disease. These interactions are impossible to accurately replicate in non-animal means at the present time, and there are no in vitro models available that can model effects on both tumour growth and bone disease.

Which non-animal alternatives did you consider for use in this project?

We have considered using cell culture, including the use of 3D models or organoids. We gain valuable information regarding the biology and drug response of individual cell types using standard cell culture techniques, and all our initial experiments are performed in vitro. We are developing methods to enable the simultaneous culture of multiple cell types found within the tumour-bone environment, however these experiments are in their infancy.

Why were they not suitable?

It is not currently possible to culture together all cell types found within the bone, and so not possible to accurately recreate and manipulate the tumour-bone microenvironment in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers of animals we will use, based on previous experience in doing these types of studies and on statistical calculations to determine the number of animals required. We have considerable familiarity with these experimental approaches and will perform pilot studies in those cases where we do not, to gain understanding of the timing of the tumour development and/or response to therapy and of the statistical variability to ensure that the smallest numbers that can yield statistical significance are used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3Rs Experimental Design Assistant to plan our experiments with the minimum number of mice. Where possible, we pool data from repeat experiments for statistical analysis which allows us to reduce the number of mice used in each repeat. Where possible, we use one control group for multiple experimental groups to reduce the number of mice required.

We will be conducting our experiments to comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines) at time of publication.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding is usually managed by support staff specially trained in maintenance and breeding of genetically altered mouse colonies. The MCMS database is used, taking advantage of the efficient breeding and notification systems. This allows us to keep the number of surplus animals to a minimum. Experimental design will be optimised by the use of MCMS study plans. Tissues are routinely shared within multiple research teams, in

order to reduce the number of animals used. Where necessary (e.g. new cancer cell line) pilot studies will be performed to gain understanding of the timing of the tumour development and/or response to therapy and of the statistical variability to ensure that the smallest numbers that can yield statistical significance are used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models that we use are well-characterised, standard and the most refined in the field. We use the most refined model that can answer the scientific question. Typically, this is a model that requires tumour growth within bone, but where possible we use a less-invasive subcutaneous model. Models are selected based upon the development of the tumour cells selectively within bone, thus eliminating the risk of tumour growth at other sites. All models are designed to have a short duration (typically 4 weeks, sometimes up to 12 weeks)i. Where it is necessary to fast animals, we will use the minimum amount of time necessary to achieve accurate measurements of the factors under investigation. For long-term or continuous drug treatments, osmotic minipumps may be implanted to reduce the frequency of drug administration. Taken together, these steps will minimise the welfare costs to the animals, while enabling us to achieve our scientific objectives.

Why can’t you use animals that are less sentient?

Mice are the least sentient animals that have anatomy and physiology similar to that of humans. The reason that tumour growth within bone and the associated bone disease is so rapid and aggressive is due to the complicated interactions between tumour cells and host cells within this microenvironment that promote both tumour growth and survival and bone disease. These interactions are impossible to accurately replicate within less sentient animals. Age is an important contributary factor to tumour development, so the use of aged animals is required to accurately reflect this.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are routinely housed in individually ventilated cages to minimise the risk of infection and supplied with environmental enrichment.

When administering agents to the mice we will use the least invasive route possible, for example administration of viscous substances by gavage rather than injection.

Observed outcomes of initial studies carried out on this licence will be used to revise endpoints in future studies.

Where needed, animals will receive pain relief and/or will be under general or local anaesthesia. Tumour growth and animal behaviour is closely monitored.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will make use of the resources available at the NC3Rs website (www.nc3rs.org.uk), including the NC3R's Experimental Design Assistant where relevant, and the Laboratory Animals Science Association website (https://www.lasa.co.uk). We will follow the ARRIVE guidelines (https://arriveguidelines.org) when planning, undertaking and publishing this work.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All members of the team associated with this licence attend local good practice and welfare meetings, and we are in receipt of regular e-mails from the University's NC3Rs manager. Attendance at the local annual NC3Rs day is encouraged. We will consult the NC3Rs website for the latest updates.

When undertaking a procedure for the first time, we will consult the vets and animal welfare officer beforehand so that any recent 3Rs updates can be implemented.

71. Remodelling of stem cells and niche cells: from development and regeneration to disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words
Respiratory system, Stem cells, Tissue repair, Ageing, Lung disease

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to investigate how the cells of the lung adapt to tissue perturbations (situations driving the tissue away from normal health condition) such as ageing, cell depletion and tumour development. I aim to learn if we can use this information to improve injury repair in lung tissues.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The lung is exposed to external environment and faces with constant challenges by various insults such as allergen, air pollution, chemicals, and pathogens which lead to tissue damage and ultimately developing lung diseases. Lung disease is one of the top three killer diseases in the worldwide. The Asthma and Lung UK estimates that approximately 12.7 million people in the UK have a history of longstanding respiratory illness including lung cancer and pulmonary fibrosis (scarring of the lung tissue). Most significantly, the recent COVID-19 pandemic, caused by SARS-CoV-2, has already recorded more than 300 million confirmed cases, while as of 18th January 2022, more

than 15 million deaths have been reported worldwide (WHO, 2022). The cells of the lung are the main target of SARS- CoV-2 infection. Further, patients with chronic lung diseases are at greater risk of developing severe COVID-19, presumably due to the unresolved lung damage or alteration already present within their lungs or an overactive immune response. Therefore, with the global scale and clinical burden of COVID-19, a deeper understanding of lung repair mechanisms following tissue perturbation (situation driving the tissue away from normal health condition) is urgently needed.

In this project, we will study the way in which the cells of the lung function under normal conditions, evaluate how their behaviour changes in response to tissue perturbations. By addressing these questions, we expect to understand how cells behave in different contexts, and whether this information can be used to change their behaviour to improve the health condition of the lung. We ultimately aim to improve lung injury repair with minimising fibrotic scarring (regeneration) and to treat disease more effectively. By investigating cellular changes from its early stages of lung disease, we expect to identify markers that can assist in the early detection of these diseases, improving the long- term prospect of those patients. We anticipate that our work will identify targets of potential medical relevance that will contribute towards solving the challenges being currently faced in the clinic.

What outputs do you think you will see at the end of this project?

The main output of our work will be in the form of scientific publications that will be of relevance to other scientists in our or other related research fields. These publications will provide novel information on the processes regulating how the cells of the lung respond when not in normal health.

In our publications, we aim to cover a number of relevant aspects:

Identify changes in the damage repair ability of cells during aging, and its implication in chronic lung disease.

Uncover how changes in the immediate environment that surrounds stem cells affect tissue damage repair.

Define how cells return to an early developmental stage when perturbed, and its implication in lung cancer.

As standard practice, the data generated in our work will be made publicly available in free repositories to ensure that it can be used by other researchers and facilitate scientific discovery beyond our own studies. This should reduce repetition of the same work by other laboratories, decreasing the number of animals needed for related research purposes.

Who or what will benefit from these outputs, and how?

Our overall challenge is to identify the processes controlling the ability of a lung tissue to heal (regeneration) and to develop lung diseases including lung cancer and pulmonary fibrosis (scarring of the lung tissue), and translate this knowledge into therapies that benefit the patients and healthcare providers. For this, the immediate aim is to understand how the cells deviate from normal behaviour when they face an injury/damage or develop disease. Then we need to determine which molecular mechanisms regulate the cellular

response. Our ultimate aim is to determine whether we can intervene in these processes, using drugs, in order to take cells back to their normal healthy state.
In this programme of work we will address these important questions. In the short term, new information that comes from this study will be presented through publications and conference presentations, or shared with organisations such as the Wellcome Trust and The Royal Society. This will mostly benefit the scientific community, particularly those with interests in tissue regeneration and chronic lung disease. In the long-term, our findings could be used to devise new strategies for the early detection of lung diseases as well as to develop novel therapies to improve lung regeneration and treat diseases. This will benefit patients suffering from declining lung function, professionals in the healthcare community, as well as companies with an interest either in i) experimental systems to study disease or in ii) in the potential identification of novel drug targets.

How will you look to maximise the outputs of this work?

We maximise the outputs of our work by doing the following:

To increase the outputs of the work conducted within this study, the management of individual project is closely supervised by me. This way I avoid effort duplication and ensure that the resources are maximised and shared between members of my laboratory, where feasible.

By disseminating our observations from early stages, for example via conferences, we create new collaborations with experts in research fields beyond our own. They positively contribute to the progress of ongoing projects and ensure their completion to the highest standards. This has previously allowed us to establish collaborations with researchers interested in samples created as a side-product of our research. For example, samples are currently being shared with other groups examining the cell behaviour in other organs such as the intestine, brain, liver, among others.

Moreover, in our effort to understand the behaviour of cells, we conduct the analysis of large- scale datasets. To maximise the outputs obtained from these complex datasets, we collaborate with experts in theoretical physics, mathematical modelling, and computational analysis, who have the skills to develop theories explaining the results obtained in our experiments. Methods and datasets generated in these types of analyses are made publicly available as part of the relevant research articles enabling new collaborative projects.

Our work will be published in free, public repositories online and in international scientific journals. In our publications, we do not only include positive data, but also experimental results that fail to support our hypotheses. This also prevents duplication by ensuring that other laboratories do not spend resources in ideas that have already been tested.

We will share our work with the public via public engagement activities. This will allow people to learn about novel aspects of regenerative medicine and lung diseases, and will provide the opportunity for a productive exchange of ideas between patients and scientists.

Species and numbers of animals expected to be used

- Mice: 41050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 mice will be used in this project for tracing the changes in behaviours of the lung cells under tissue challenges (conditions away from healthy normal lungs). A crucial reason for using mice is that cellular responses to tissue challenges represent a complex biological process that involves numerous cellular types, signalling pathways and environmental factors. These processes can differ substantially in organisms other than mammals and, specially, in invertebrates. Mice are the smallest species with lowest susceptibility to neurophysiological conditions (situations that affect both physical and conscious functions such as stress, anxiety, and mental activity) that have mammalian lungs. Moreover, stem cells that contribute to lung tissue maintenance have been extensively studied by using multiple mouse models that are readily available and capable of reproducing the human settings. Critically, at the moment, there is a critical limitation to investigate the time-course changes of disease initiation and progression for which human samples are typically unavailable yet mouse disease models are allowed. Our study will focus on the early sequential changes of lung cells upon tissue challenges using mouse models which also allow relatively easy genetic manipulation and tracking the changes of individual cells over time.

We will use mice that undergo different stages of their lifetimes (from birth to aged stages) because we are interested in understanding why we exhibit a different repair response and disease incidence as we age.

Typically, what will be done to an animal used in your project?

Most of our experiments will use genetically modified animals that allow us to follow the individual cells in which we are interested. This will facilitate the tracking of individual cells to study their behaviour in response to perturbations (such as ageing, genetic mutation, or cell ablation). To address our research questions, animals will typically undergo one of three main types of experiments:

Ageing: Animals will be administered various substances to label their cells, by delivering them into their stomach. This will help us track the behaviour of cells at different time points throughout the lifetime of an animal. Samples will be collected after humanely killing mice at different ages from birth and up to 24 months of age. The labelled cells will be analysed to investigate the impact of ageing on cells.

Genetic mutation: Animals will be administered substances to alter genes that are associated with lung cancer, by delivering them into their stomach. Animals will then be allowed to develop tumours in the lung. Since we are interested in early tumour formation, animals are humanely killed before showing any signs of distress or suffering. Tumour samples will be collected at different times to capture the point at which tumours form. The cells will be analysed to investigate how tumours emerge.

Cell ablation: Animals will be administered well-studied substances for local cell ablation in the lung, by delivering them into abdomen or lung. The procedure of lung delivery will be completed under inhalation-mediated anaesthesia for less than 5 minutes so that animals remain in a state of sleep/unconsciousness during the procedure. Some animals may develop mild scar (fibrosis) after cell ablation in the lung. Some animals may be treated with additional substances to label the cells and track their behaviours during

replenishment process of lost cells or development and progress of lung fibrosis. Aged animals may be used to compare these features to the young animals. Subsequently, samples will be collected after humanely killing mice at different stages of replenishment or fibrosis to investigate the sequential changes in cell behaviour. These experiments will shed light on how tissue repair changes as animals age.

For any of these typical experiments we may sporadically treat a small number of animals with drugs that will help us discover the processes that regulate cellular behaviour.

What are the expected impacts and/or adverse effects for the animals during your project?

Please note the adverse effects presented here are based on our previous experience using the proposed techniques. Our work will be conducted in a way that will not lead to long-lasting pain, suffering or distress and have no lasting effect on the animal's health and wellbeing. The genetically modified mice used are not expected to show deviations from normal health.

All animals are carefully checked regularly and if there are any concerns animals are examined and weighed. The majority of the animals will be used in procedures under which they suffer either no, or mild, adverse effects. For example, transient pain or discomfort following an injection. A smaller number of animals (less than 5% of the total) will be exposed to procedures of moderate severity to model different aspects of human lung disease. Some animals sporadically present transient weight loss of up to 15%, which is usually recovered within the next 2-7 days. In some instances, animals become sick from unresolved tissue damage or scarring that causes sustained altered breathing rate and subdued activity, in which case they 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 animal type)?

Mice.

-Mild: 67%

-Moderate: 33%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal experimentation is required to obtain relevant information on cellular behaviour within the living organism. In order to identify treatments of relevance to improve injury repair and the symptoms of illness, we need to understand the way in which different cell types interact with each other. The lung is complex organs formed by different layers and tissue compartments that contain different types of cells. In response to an injury or genetic alteration leading to lung disease, all these cells need to communicate in order to elicit a coordinated response that results in injury repair or disease development, respectively. Unfortunately, this complex communication network is something that so far cannot be fully explored merely by growing cells outside the animal. Additionally, most techniques that work with cells outside of living animals do not fully reflect their normal behaviour over long periods.
Hence, animal work is necessary to disentangle how different cells communicate and cooperate in health and disease; an aspect of critical importance to understand the basis of tissue regeneration and cancer.

Which non-animal alternatives did you consider for use in this project?

My laboratory is a pioneer group in developing methods, called three-dimensional (3D) organoid systems, to grow tissues outside the animal. These platforms recapitulate physiological features of lung tissues in a dish and thereby significantly replace injury/disease animal models that generally employ moderate to severe animal protocol. Additionally, we have published the details of our methods which can be used by other scientists who can also replace their animal models. Importantly, we have been educating more than 500 scientists from academia and industry to grow 3D lung organoids in a day workshop. Whenever possible, we will implement this technique to obtain preliminary data to inform experiments with animals in this project.

Why were they not suitable?

Tissues are formed by many different types of cells. We are interested in understanding how those different types of cells interact with each other and contribute to ageing, injury repair and disease development over long periods of time. Unfortunately, to date, by growing cells/tissues outside the animals, we are not able to fully mirror the complex cellular interactions and time-course long-term changes in cell behaviour that take place in the animal. Until then, experimentation with animals will still represent the gold standard to unveil these intricate 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?
The predicted number of animals needed for this project has been based on the following:

Animal numbers have been worked out with the advice of expert statisticians. This ensures that our experiments are designed properly and that the results obtained have enough statistical power to draw meaningful biological conclusions.

Animal numbers have been calculated based on the animals used in my laboratory in similar work over the past 5 years. I have also accounted for an increase in the number of members that form my laboratory, something anticipated for the next five years.

We have carefully considered the best way to make sure we keep the lowest number of different types of mice (strains) for breeding, while ensuring we have enough mice to use for experiments.

Test experiments, called pilot experiments (which use a smaller number of animals; typically 3) will be used to calculate the amount of a substance that we can safely inject into the animal. It is important to find the smallest amount of the substance that has an effect but that will not harm the animal in any way. This will then allow us to perform the intended experiment using only enough mice to be sure that the results we obtain are statistically significant.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken advice received from a local, qualified expert in statistics. This will make sure that each experiment produces statistically meaningful data. Where possible, all experiments are designed to get the most information using the least number of animals possible. We will also take into consideration the NC3Rs guidance and experimental design assistant tool (https://www.nc3rs.org.uk/experimental- design-assistant-eda; https://nc3rs.org.uk/3rs-advice-project-licence-applicants-reduction). Additionally, all experiments will be designed taking into account the PREPARE guidelines (a document that gives scientists advice on how to plan animal research and experiments).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We implement different measures to reduce the number of animals used. Among them:

Efficient breeding techniques: We will breed our genetically modified mice in a way to minimise waste. Any types of genetically modified mice (strains) that are not being used for scientific studies will be frozen as embryos for future use.

Experimental design: Critical experiments are designed in collaboration with experts in statistical physics. With their assistance, we determine the number of experimental animals required to answer each of our scientific questions.

Mouse strain management: animals are bred only to fulfil our experimental needs. However, any unavoidable excess of animals is used for in vitro experiments (culturing cells outside the animal), validation or pilot studies (as long as they are compatible). The latter represents a critical consideration in our laboratory, and allows us to ensure that mouse waste is minimised as much as possible. While this approach requires a significant amount of coordination and team effort, it is essential to reduce the number of animals we breed or purchase. We will also take into consideration the NC3Rs Breeding and Colony Management Resource (https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management).

Animal and tissue sharing: I have a network of collaborators within the UK who work in my or a similar research field. We exchange particular types of mice (strains) between us to reduce animal imports, the need for additional breeding and its associated excess.

Similarly, to increase the output of our work, we share tissues with members of other research groups and collaborators who examine different areas of the body (tissues), such as the liver and the intestine. Additionally, we are registered in the animal tissue sharing list of our institution.

Growth of tissues outside the animal (3D tissue cultures): Using this method, one single lung can produce sufficient cells to perform at least 3 experiments instead of one. Thereby, this system effectively reduces the number of animals used by two thirds. However, as indicated above, studying interactions between different types of cells remains a limitation when using this technique.

Mathematical modelling: By working with our collaborators, we use our data to develop mathematical models that explain how cells behave and interact with each other. The created hypotheses are then tested in the laboratory. This makes our science more targeted, which allows us to significantly reduce the number of animals needed to understand how cells work.

Advanced imaging and molecular methods: Dissected samples are analysed using state-of-the- art whole-tissue imaging and molecular techniques that require minimal tissue material to obtain meaningful results.

We will also consider subscribing to ATLA (Alternatives to Laboratory Animals) published by FRAME (Fund for the Replacement of Animals in Medical Experiments), in association with SAGE publishing, featuring articles on the latest research relating to the development, validation, introduction and use of alternatives to laboratory animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically modified (GM) mice to carry out our plan of work. Most of our GM mice bear genetic alterations that allow us to label cells and track their behaviour in the tissue. Hence, the majority of genetic alterations we propose are not expected to lead to any adverse effects, except animal models for early tumour formation (please see below).

The animal procedures proposed in this study will inflict minimal pain, suffering or distress to mice:

We administer substances to mice to label and track cells over time. These methods are based on previous studies and our long-term expertise using them. We use the lowest dose needed to observe an effect. Hence, we expect our treatments to cause no harm to animals.

We also use GM mice bearing lung cancer-associated genes to understand how cells work and explore the earliest stages of tumour formation in the lung. Our project mainly focuses on the early tumour development and use well-established GM model system based on our experience and published literature. Therefore, mice rarely show complications apart from developing discrete lesions, and will be killed before the tumours have an impact in any vital process.

Animals receive drugs causing localised short-lasting damage in the lung. These methods affect the limited region of the lung and ablate specific cell types which are typically replenished within 1-2 weeks, depending on cell types and methods. Therefore, effect is both refined and only short- lasting. Animals undergo inhalation-mediated anaesthesia which allow a rapid recovery (less than 5 minutes) from procedures and unconsciousness during the procedure.

Why can’t you use animals that are less sentient?

In our studies, we investigate how different cell types function under normal circumstances, and how they change their behaviour in response to injury and early disease development. Given the differences observed in injury repair and incidence of lung disease, such as lung fibrosis and cancer, as we age, it is important to investigate cell behaviour over long periods of time and at different stages throughout the life of animals (from birth to aging). Unfortunately, these long-term experiments require living organisms, limiting the use of other less sentient animals such as anaesthetised mice or tissues grown outside the animal.

Another critical aspect of our work focuses on investigating how different cell types interact and impact on each other's behaviour. For these studies to be clinically relevant, they need to be performed in mammalian animals, closer to humans. Results in lower species such as fish, worms or fruit flies, would be of very limited medical relevance.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All methods in this project will use techniques that reduce animal stress and make sure the animal does not suffer as detailed below:

General:
We will make sure to use the best care methods to improve the quality of life for the animals. Mice will be placed in cages and will be provided with nesting and bedding material to stimulate mouse activity and provide them with shelter if needed.

Side effects from procedures will be monitored by regularly weighing animals, daily health checks, and the use of scoring sheets, as required. This will prevent animal suffering. Animals showing any signs of suffering will be immediately killed. This is called the Humane Endpoint. We are not expecting animals undergoing procedures under this licence to experience suffering. If an animal does begin to look unhealthy, we will monitor it more frequently and provide pain relief if needed. If it does not improve the symptoms of illness the animal will be killed by a humane method.

Some substances have to be given to animals by injecting them in specific places. We have therefore asked to be allowed to use different administration routes (e.g. intraperitoneal [injection into the abdomen], oral [via the mouth] and intratracheal [into the lung] in this project). We will always use the least harmful route possible to give an animal

a substance. This is to make sure we cause the smallest amount of discomfort or pain to the animal.

All animals that are brought into the animal facility will be allowed at least 7 days to get used to their surroundings. This process is called acclimatisation. We will also allow them to get used to the animal technicians prior to use. This will reduce the amount of stress the animal experiences and will improve their well-being.

To improve the quality of life of our animals, we always house them in groups, unless strictly necessary due to experimental reasons or unexpected husbandry issues such as fighting.

Animals have enrichment in their cages (such as wood sticks, bedding and nesting material) for extra comfort and enhanced mental and physical health.

We will carry out pilot studies when new substances or genetically altered (GA) mouse lines are used for the first time. These preliminary experiments will be guided by the relevant literature.

Conditions are first validated in reduced cell culture pilot studies using less than 3 animals to perfect the experimental design required to satisfactorily address a particular experimental question.

Surgery procedures:

Surgery will be carried out in a clean manner (using aseptic technique). We will make sure to meet the level set out in the Home Office Minimum Standards for Aseptic Surgery and the LASA Guidance on Preparing for and Undertaking Aseptic Surgery (2017).

We will ensure that animals suffer as little as possible during surgical procedures by giving the animals medication to manage their pain under the advice of the Named Veterinarian Surgeon. We will also consider the use of medicated palatable substances for voluntary treatment such as flavoured jelly, paste or milk shake liquid.

Animals will be monitored before and after surgery to ensure that any deviation from normal health is picked up. Observations will be monitored on a chart. Animals will receive additional pain relief medication as needed, and advice from the Named Veterinarian Surgeon will be sought if animals show any deviation from normal behaviour.

Surgical procedure will be completed under inhalation-mediated anaesthesia for less than 5 minutes so that animals remain in a state of sleep/unconsciousness during the procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the guiding principles on good practice for Animal Welfare. Our experiments are planned following the "Planning Research and Experimental Procedures on Animals: Recommendations for Excellence" (PREPARE) and "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) guidelines. We attain to the LASA (Laboratory Animal Science Association) guidelines, as well as the NC3Rs published strategy for improving animal welfare (see publications details below):

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.). http://www.lasa.co.uk/publications/

Smith D, Anderson D , Degryse A, Bol C, Criado A, Ferrara A, Franco NH, Gyertyan I, Orellana JM, Ostergaard G, Varga O, Voipio H (2018) Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Animal 51(1S): 5-57. doi: 10.1177/0023677217744587

Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T (2018) PREPARE: guidelines for planning animal research and testing. Lab Animal 52(2): 135-141. doi: 10.1177/0023677217724823.

For surgical procedures we follow the Laboratory Animal Science Association (LASA) Guidance on Preparing for and Undertaking Aseptic Surgery (2017) and the Home Office Minimum Standards of Aseptic Surgery.

For the breeding of genetically altered mice, we will follow the guidelines provided by the Home Office and the NC3Rs Resources on 'Genetically altered mice' detailed in:

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_da ta/file/773553/GAA_Framework_Oct_18.pdf

https://www.nc3rs.org.uk/GAmice

Additionally, for cancer studies, we will refer to:

Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double AJ, Everitt J, Farningham DAH, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA, Committee of the National Cancer Research Institute. Guidelines for the welfare and use of animals in cancer research (2010). Br J Cancer 102(11): 1555-1577. doi: 10.1038/sj.bjc.6605642.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

At our institution, we count on with the outstanding support of our Biofacility Service. They keep us informed (through their central team) about new developments on 3Rs and offer us expert advice on how to implement them in our ongoing studies (via our very experienced team of animal technicians, Named Animal Care & Welfare Officer (NACWO), Named Veterinary Surgeon (NVS) and Named Information Officer (NIO) all of them experts in animal experimentation).

We follow the website of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs, available at https://nc3rs.org.uk/resource-hubs). This allows us to stay up-to-date on the most relevant information.

We also use our Institutional 3Rs search tool. This contains an up-to-date database with information on the best ways to reduce or replace animals in our experiments. It also contains advice on how to refine methods in order to reduce animal stress.

Additional guidance and information on the most appropriate and refined techniques for our studies may be obtained from external sources, including:

Laboratory Animal Science Association (LASA) Institute of Animal Technology (IAT)

Norecopa (https://norecopa.no/databases-guidelines) Relevant literature

ARRIVE Guidelines

72. The role of the RASSF family in 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

tissue architecture, development, cell polarity, tissue growth, regeneration

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to understand how the complex architecture of tissues and organs is built during animal development. In particular, we will study the RASSF (Ras association domain family) proteins, which have been implicated in this process, by generating knockout mouse models for these genes and studying the impact of these genes on development and tissue regeneration. We are particularly interested in how cell identity and polarity are precisely orchestrated in highly polarised organs such as the placenta, skin and kidneys.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 build functional organs, each cell within an animal has to adopt the right fate,

shape and orientation to yield the appropriate tissue architecture. This organisation has to be maintained during adult life, and restored after injury through tissue repair. Normal tissue organisation is essential to prevent cells from becoming cancerous and disrupted tissue architecture is a hallmark of cancer.
Studying how tissues are organised during development and maintained in adult life is therefore key to understanding not only how complex organs function, but also how to prevent tumour formation.

What outputs do you think you will see at the end of this project?

The main objective of our work is to generate new knowledge and promote a better understanding of how complex organs are built during development and maintained throughout adult life. The primary output will be publication of our results in peer-reviewed journals and deposition of raw data in the appropriate repositories to make our findings available to other researchers to build on.

Our work will also generate new lines of genetically altered mice that can be distributed and used by others in the field, for future research.

Who or what will benefit from these outputs, and how?

In the short term, new knowledge on the mechanisms of organ development will be beneficial to scientists working in developmental and stem cell biology.

In the longer term, we anticipate our findings will inform future work on several disease states. Unravelling how normal development proceeds is essential to understand how organ function fails in pathogenic conditions. For instance, our work on placenta development will promote a better understanding of how placenta failure or insufficiency occurs, which is a major cause of complications during gestation. Given the strong link between tissue architecture and cancer, as well as the genetic evidence pointing at a role for the RASSF family and the Hippo pathway in cancer formation, we also anticipate that our work will benefit the cancer research community. For instance, although the RASSF family is frequently lost in a range of human tumours, there is little insight into why these genetic losses are favourable for cancer formation. Understanding the consequences of RASSF loss in tumours might lead to improved therapeutic options. Finally, our work on tissue regeneration may lead to improved strategies for tissue repair.

How will you look to maximise the outputs of this work?

In order to maximise outputs from this research, we will ensure the timely publication of our findings and we will promote our work on social media and by presenting it at international research conferences. To make our work available as early as possible, we will release pre-peer reviewed versions of manuscripts on appropriate preprint servers such as bioRxiv upon first submission to peer-reviewed journals.

Through internal and external collaborations, we will share both positive and negative results and best practice with our peers. We will share our mouse transgenic lines or tissue extracted from these models with others in the field in order to accelerate the progress of research and reduce overall animal usage.

Species and numbers of animals expected to be used

- Mice: 15600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our aim is to investigate how complex organs develop in mammalian systems, therefore mice represent the best available model, due to the many similarities with human development and the wealth of genetic tools available. The majority of our work will be performed during embryonic development and we will endeavour to focus on the earliest developmental times where a phenotype becomes manifest. For our work on tissue regeneration, we will also need to use adult animals, as the mechanisms for tissue repair during embryonic development and in adult animals are radically different.

Typically, what will be done to an animal used in your project?

Most of our experiments will involve the breeding of genetically altered animals, both for generating experimental animals and for maintenance of genetic lines for future experiments. For experimental animals, breeding will be followed by injection of a gene expression-modifying reagent (tamoxifen or doxycycline) and/or tracer dye if necessary. Upon reaching the appropriate stage, the animals (and the mother in the case of embryonic stages) will be killed and tissue will be harvested either for analysis (e.g. immunohistochemistry) or placed in ex vivo culture conditions.

For a small number of animals, adults of the appropriate genotype will undergo a standardised technique to create a small (4mm) shallow wound in loose skin on the back using a sterile biopsy punch. The aim of these experiments will be to evaluate the role of RASSF family members or functionally related proteins in tissue regeneration. Analgesia will be provided and the animals will be closely monitored to ensure their full recovery.

To generate new transgenic lines if necessary, a small number of animals will undergo surgical procedures to transfer embryos into the uterus of adult female mice. These animals will be closely monitored, and anaesthetics/analgesics will be used as appropriate.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the phenotypes will be analysed at embryonic stages, less than two thirds into gestation, when the animals are not considered sentient. We will generate genetically modified animals, the vast majority of which are expected to display either no phenotypes or moderate phenotypes such as hair loss.
Animals undergoing the punch wound or embryo transfer protocols will experience mild to moderate post-operative pain which will be controlled with analgesics. Full recovery is expected within one or two 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)?

Moderate severity is expected for up to 14% of animals. The rest of the animals will reach a maximum of mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our project aim is to understand the precise cellular interactions that allow the development and maintenances of highly complex organs. In order to tackle this question, we have used and will continue to use simple models such as fruit flies, mammalian cells in culture, computational models and different types of ex vivo cultures systems such as gastruloids. However, in order to fully recapitulate the complexity of organ development, it is necessary to observe how this occurs in an animal model, as there are currently no cell culture models that fully reconstitute this process. Since ultimately our aim is to gain insights into human development, mice are the in vivo system of choice due to the close similarities between mice and humans during development and the number of genetic tools available in the mouse system.

Which non-animal alternatives did you consider for use in this project?

As outlined above, we have used several less complex systems (fruit flies, cell culture, computational simulations) to minimise the number of vertebrate animals used in our experiments. We will continue to monitor the rapid progress in ex vivo differentiation and organoid models with a view to incorporating as many of these emerging technologies into our research as possible.

Why were they not suitable?

Cell culture-based models such as blastoids and gastruloids are showing a great deal of promise to study development, particularly its early phases. However, these models remain imperfect and fail to capture the complexities of organ formation, which involve complex interactions between many different cell types, and in many cases communication between different organs. One example relevant to our work is the interplay between the mother and embryo during the development of the placenta. Although we will take full advantage of cell culture, it remains necessary to use animals to capture the real complexity of development.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our animal use projections are based on several factors:

Number of genetically modified lines we are currently maintaining.

Complexity of the crossing schemes to generate experimental animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We carefully reviewed the cross schemes required to generate the desired genotypes in order to ensure we use the minimal number breeding steps to generate the maximum number of experimental animals. We regularly review our mouse colony and freeze sperm and embryos in order to archive lines that are not immediately required. To achieve efficient colony use, we work closely with the mouse technicians in our animal unit and our institution's colony management team. Our institution also supports sharing of useful genetically modified animals such as Cre lines with other licensees in order to minimise animal usage at the institutional level. This is enabled by our sophisticated electronic colony management system.

The RASSF gene family contains 10 members, which likely perform redundant function, making the analysis of all possible mutant combinations using standard genetic approaches hugely costly in terms of animal breeding. To circumvent this problem we will screen for phenotypes in mutant combinations using mouse embryonic stem cells and in vitro differentiation protocols such as gastruloids. This will allow us to screen for combinations that elicit phenotypes prior to generating mouse lines and will therefore allow us to focus only on informative combinations. This approach will considerably reduce mouse usage for this project. Whenever possible, we will use established protocols to derive stem cells from mutant and control animals, and differentiate them in vitro into the cell types we want to study. For instance, to support our work on placenta development, we will derive trophoblast stem cells and differentiate them in vitro to compare gene expression profiles between mutant and control animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In addition to the measures outlined above, when we generate genetic combinations for analysis, we will maintain as many of the genetic elements as possible in the same line without causing harmful phenotypes in order to minimise the numbers required to generate experimental animals. Where we generate a new mutant line to investigate its phenotype, we will perform pilot studies with a small number of animals in order avoid generating a large cohort that will not present any phenotype we want to study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen to use mice for these studies for a number of reasons. They are ideal model organisms to investigate mammalian development and regeneration - their biology is close enough to that of humans for our findings to be relevant to human disease. Mouse embryonic development and biology are well described, so we will be able to identify and characterise abnormalities easily. As our primary interest is development, we will seek to study phenotypes at early developmental stages where the animal is less sentient in order to minimise suffering. Our analyses will primarily be performed on dissected tissue following the killing of the subjects via humane methods.

To study regeneration, we have chosen a punch wound protocol that induces relatively little discomfort compared with many other injury models such as liver regeneration upon partial hepatectomy or toxic substance ingestion. This protocol was chosen because the Hippo signalling pathway, which we are investigating, has been shown to affect epidermal regeneration using this experimental system.

Why can’t you use animals that are less sentient?

Most of our work will be carried out on embryos prior to two thirds of gestation, which are not considered sentient. Wherever possible, we will derive stem cells from our animals in order to reduce the number of animals we need to breed for our experiments (e.g. trophoblast stem cells for out placenta work). For our regeneration work, we need to use adult animals as the wound healing process is very different in adults compared with embryonic stages. Our work on mice will continue to be guided by our work in fruit flies and cell culture, which allows us to reduce mouse usage.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our close collaborative relationship with the staff in our animal facility ensures that animals having undergone procedures such as our epidermis regeneration protocol are closely monitored. New genetic combinations which may develop unanticipated phenotypes will also be closely monitored. In these cases, the animals will be monitored daily or twice-daily depending on the situation. Animals exhibiting unexpected harmful effects will be killed using an approved method, except in rare cases where these animals are essential for an experiment, in which case we will seeks advice from the Home Office Inspector.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow guidance and best practice provided by the NC3Rs, as well as our animal unit and scientific colleagues.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our animal unit communicates best practice and new advances in animal handling through regular newsletters and annual refresher sessions for project and personal license holders. In addition, we will keep abreast of new advances in ex vivo culture experiments through conferences and publications.

Finally, we will regularly monitor the NC3R web site for updates (https://www.nc3rs.org.uk/3rs- resources).

73. Determining what happens to intestinal T cells

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

T cells, Intestine, Memory, Infection, Inflammation

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's the aim of this project?

The aim of this project is to determine the nature of the memory T cell populations that are formed following intestinal infection. We will define what these cells are doing, what they are making, whether they are migratory and how they contribute to local (intestinal) versus systemic (the rest of the body) immunity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 immune systems are taught to distinguish between molecules encoded by our own DNA (termed 'self') and everything else (termed 'foreign'). Immune cells are therefore poised to respond to all foreign molecules in our body, whether they come from the food we eat, harmless bacteria that colonise different surfaces in our body or disease causing organisms (bacteria, viruses, fungi, collectively termed 'pathogens') that enter our body. The immune system is able to establish an acceptance of certain molecules that do not cause us harm, which is termed 'tolerance'. In responding to organisms that cause disease, our bodies seek to establish 'immunological memory' which refers to an improved ability to fight off a previously encountered infection and underpins vaccination. The mechanisms that dictate whether a state of tolerance is formed versus responses that

generate memory populations are key to maintaining normal functions within the body as well as keeping us healthy.

The intestinal tract is largest surface in our bodies at which we encounter foreign molecules and so the immune system is constantly active, suppressing responses to everything that is harmless (e.g. food and commensal bacteria) while suitably responding to pathogens seeking to enter the body to prevent infection. To achieve this, what happens to different immune cell populations (their ‘fate’) must be carefully controlled. While we know some of the details of this process, details of what happens to key immune cells called 'T cells' during this process remains unclear.

Crucially, when the regulation of immune responses in the intestine doesn't work properly, patients suffer intestinal inflammation. This is very common, can be life-long and highly debilitating and affects nearly 7 million people in the world. This inflammatory state is associated with an expanded population of T cells with altered functions, suggesting that something has gone wrong in regulating appropriate T cell responses and the formation of memory populations. Why these abnormal T cells have formed and how best to either control these cells, or the processes regulating their formation needs to be worked out. Therapies that seek to limit intestinal inflammation remain a work in progress, successful in only a proportion (~50%) of patients, meaning that there is a huge need for other treatments.

Through defining how different intestinal T cell populations form and what happens to them over time, basic research can help identify pathways that should be targeted in new therapies. Importantly, understanding the regulation of intestinal T cell responses is highly relevant to oral vaccination. This represents significant potential refinement to current injectable vaccines, particularly in terms of ease of delivery and required expertise, storage and distribution in less developed countries. Importantly, a lack of understanding of the memory populations that form following intestinal exposure to antigen and how this is controlled, undermines the development of oral vaccines.

In this Project we will determine what happens to the T cells generated in response to intestinal infection, how and why this affects their functions and what this means for our immune system. T cells lie at the very heart of our immune system, coordinating what many other cells in the immune system do. While our research is at the basic discovery stage, through defining the fate and function of these cells in fine detail, alongside mechanistic insight into how this is controlled, our research can influence the development of new therapies and better inform efforts to develop effective oral vaccines.

What outputs do you think you will see at the end of this project?

The key outputs of this project will be

a detailed understanding of what happens to T cells after intestinal infection, determining the types of memory cells that are formed, what they do and what kind of protection they can provide. This information will be published in scientific journals and potentially influence the design of oral vaccines.

detailed characterisation of the inflammatory T cells that are formed, why this occurs and what this means for the health of the intestine. This information will again be published in scientific journals and will support efforts to better disrupt the generation or persistence of inflammatory T cells in patients.

We have consistently published all our findings in high impact immunological journals and we will continue to do so as the best way of advancing understanding from our research. In addition we will continue to present our research findings at scientific conferences, invited seminars, interactions with industrial partners and public engagement events.

Who or what will benefit from these outputs, and how?

Short-term benefits: Our detailed in vivo research will directly benefit researchers, particularly mucosal immunologists, seeking to better understand intestinal inflammation and how to best limit or control this. It will also benefit researchers including immunologists and vaccinologists investigating how and why memory cells are formed from intestinal responses and how the factor that control these processes can be best manipulated. In addition to academic research, research teams within industry, e.g. pharmaceutical companies, can exploit our research findings to refine their efforts to manipulate key pathways operating in the intestine.

Medium and long-term benefits: Our research outputs and observations will feed through into both academic and industrial efforts to develop anti-inflammatory therapies targeting the intestine. This will feed through into better therapeutic options for patients with inflammatory bowel conditions and potentially other inflammatory conditions that are linked to intestinal health (e.g. rheumatoid arthritis). Through better defining the mechanisms controlling how different memory cells are formed in response to intestinal infection, our research can influence the refinement and improvement of oral vaccines, which ultimately can have wide reaching benefits to humanity through improved vaccine uptake.

How will you look to maximise the outputs of this work?

Throughout my research career I have actively collaborated with many researcher in my field and shared expertise, research tools and models widely and prior to publication. I think this is an essential part of maximising the outputs from basic research. I will continue to do this and further continue to instil this philosophy in the researchers of my lab. Publishing our work in high impact journals with broad readerships provides an effective means of disseminating experimental data and findings. I am also very active in presenting research findings from my lab nationally and internationally through invited seminars and conferences. Finally, I have established collaborative interactions with pharmaceutical companies and will continue to develop these to maximise the influence of our research activity.

Species and numbers of animals expected to be used

- Mice: 12,700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project we will use mice to further our understanding of what happens to T cells after intestinal infection. The intestinal tract is far too complex to accurately model through non-animal methods currently available (e.g. in vitro cultures). This is because not only are

there many different immune cells that must be considered due to their interactions, these cells also move between different areas of the tissue and even between tissues, which further changes what they do and why. On top of this, the epithelial lining of the intestine plays a key role in controlling the response and furthermore, all of this is influenced by the commensal bacterial populations of the gut. In summary, such a collection of cells, which exist in their own dynamic microenvironments cannot be recreated with the complexity required using cell culture systems. This really means that the only way to control for all these parameters is to study what T cells do within the intestine of a living animal. This ensures that the data we acquire is of maximum relevance to human patients suffering from intestinal inflammation and the better development of oral vaccines.

We have chosen mice as our animal study for the following three key reasons:

The main components of the mouse immune system shared by humans, making the mouse an excellent model of the human immune system.

The mouse immune system has been extensively studied already, meaning that there are an extensive range of reagents available to help careful study of what mouse immune cells do and why. This is important because it means we can use pre-existing data, we don't have to validate evidence in a new species and our research is not restricted by a lack of experimental tools.

There are many genetically modified mice available which enable detailed information about what cells do or how they behave. This is hugely important, particularly in this Project as it means we can understand cell functions, which cannot be captured by other approaches - for example tracking the movement of cells within the intestine and between the intestine and other tissues. The immune system alters over the course of development, and our research is focussed on functionality in the adult. For this reason we will use adult mice.

Typically, what will be done to an animal used in your project?

In a typical experiment on this licence, mice will be infected via oral gavage with a natural mouse bacterial infection that colonises the intestine. Most infected mice will additionally be injected (typically by intraperitoneal injection) with reagents (maximum frequency of daily, maximum number of 14 injections) to try to manipulate the response to the infection. The bacterial infection lasts approximately 3-4 weeks and is then naturally cleared by the mouse.

In some experiments we would test whether the initial infection had successfully caused the formation of a memory response by re-infecting the mice 6 wks after the initial infection, again by oral gavage and testing whether they make a better response. During the experiment we would measure how well the mice were controlling the infection by analysing bacteria in the mouse faeces.

In other experiments, we will induce intestinal inflammation to model that experienced by IBD patients. TO do this mice will be given water containing 3% dextran sulphate sodium which causes damage to the lining of th intestine and induces local inflammation. Mice undergoing this procedure will experience up to 2 periods (each of 1 wk duration) of drinking water containing 3% dextran sulphate sodium.

In some experiments we would directly assess the intestine using a mouse endoscope inserted into the rectum of the mouse while it was anaesthetised. This would typically only

be done once. Using this endoscopic approach we use light to trigger a label in the intestinal cells so that any subsequent movement into other tissues can be identified post-mortem. Mice will only undergo this labelling approach once. The vast majority of experiments would last for less than 8 weeks and at the end all animals will be humanely killed and tissues taken post-mortem for analysis'

What are the expected impacts and/or adverse effects for the animals during your project?

All mice on the project will experience temporary discomfort and stress from the handling and process by which the mice are infected. Our typical intestinal infection model does not cause overt clinical symptoms and is naturally cleared by mice without inducing adverse effects such as pain from intestinal inflammation. In mice that are given drinking water containing 3% dextran sulphate sodium, these animals will experience local inflammation in the colon and will suffer weight loss (typically up to 10% body weight). Mice on experimental protocols will continue to experience intermittent stress from restraining them (for injection) and discomfort from further injections, which we will keep to the minimum required and the least painful injection route. Mice undergoing endoscopy will do this under anaesthesia, limiting stress and discomfort from this procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum expected severity on any protocol is moderate. For approximately 60% of the mice this reflects the cumulative effect of several interventions which individually would be considered mild (e.g. infection by oral gavage, intraperitoneal injection, re-infection) but as the mice will experience these interventions several times an overall severity of moderate is appropriate. Approximately 20% of the mice will experience moderate severity where we will induce intestinal inflammation. Approximately 20% of the mice are expected to only experience procedures of a mild severity, through only a very limited number of interventions.

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 investigate what happens to T cells following intestinal infection, which includes understanding of what cells they become, where they go, what they do and why, it is impossible to study this without study a living animal and the different tissues and conditions that exist. The intestinal tract is hugely complex mixture of many cell types (immune cells, epithelium, commensal bacteria) and these cells constantly interact and regulate what happens to each cell type. Furthermore, the majority of these cells are not

static and both move between areas of the intestine and even between the intestine and other tissues. In addition other parameters change regularly over time (e.g. nutrients, metabolites) in relation to the 'body clock'. It is not possible to effectively model this environment nor the cells within it in a way that captures even a fraction of the normal cellular behaviours and interactions. Findings from only non-animal experiments would still then require extensive validation in an animal model before there relevance to human patients could be assessed and confirmed.

Which non-animal alternatives did you consider for use in this project?

We do exploit in silico (computer-based) approaches to capture existing information from previous studies. With the advent of sophisticated cell sequencing and bioinformatics approaches, which we will continue to use throughout this project, in silico studiesform the major non-animal approach we use. These experiments can provide clear direction in which molecules to try to manipulate to control what happens to intestinal T cells.

We also considered organoids, which can enable the study of single cell types in a more 3D environment and even multiple cell types for example intestinal epithelium organoids. We further investigated 3D culture approaches using a 'framework' approach where cellular interactions are enhanced. One type of cell culture approach, where we push T cells into different developmental states can be used to initially test potential interventions before some are then validated in mouse models.

Why were they not suitable?

We concluded that neither organoids nor 3D culture models would be appropriate as they completely fail to accurately model so much of the intestinal environment. Organoids cannot recapitulate the intestinal architecture, which we know has huge influences on the immune response. Currently, no in vitro model (3D culture nor organoid) can recreate the complexity or dynamism of the intestinal immune system. Furthermore, this approach would ignore the microbiome, which dramatically impacts the intestinal system and many bacterial species found in the gut cannot be cultured in vitro. Modelling all the communities of cells within the intestine and how they interact is currently not possible.

In silico models are excellent at capturing descriptive data and we will continue to inform our in vivo projects where possible, but they do not provide the means to test 'cause and effect' nor determine functional outcomes. We will screen initial candidates using in vitro T cell cultures and this will enable us to rule out some pathways/approaches that do not function as predicted from the in silico approaches.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In calculating the number of mice we will use, I have reviewed data over the last 10 years of my animal returns on my existing and previous PPLs. The number here reflects the

need to maintain an extensive breeding colony to generate sufficient experimental mice across multiple genetically modified strains.
Furthermore, efforts to reduce experimental variability, for example through using age and sex- matched animals within an experiment, or the correct use of littermate controls in conditional knockout mice experiments, means that a large colony of mice for each strain is required to ensure that sufficient numbers of the desired mice are available. For individual experiments, the number of animals required is calculated based on data obtained from previous experiments, pilot studies, and information in the literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In designing this experimental body of work, we considered our previous experience of experimental models to investigate T cell responses and have also utilised the NC3R's Experimental Design Assistant. It is our extensive experience in using the approaches described here that really enables us to keep the number of mice used in this project to a minimum. We have selected only those experimental models that gave the most robust and quantitative analysis of the intestinal T cell response and eliminated other experimental approaches where there was greater experimental variability or less clear means of definitively testing T cell function.

Through extensive research in the lab we have further optimised our understanding of the infection models underpinning this project and identified the key time points that need to be assessed. This reduces the number of analysis points when mice might be killed. Furthermore, we have developed robust means of monitoring the infection over time through counting bacteria within the faeces and extensively validated this approach. This means that infections can be tracked over time through a completely non-invasive (faeces sampling) method, eliminating the need to kill mice at specific time points to capture information regarding the progression of the infection. Through establishing and sustaining a productive collaboration with industrial partners, we are able to acquire reagents that have been extensively validated in vivo and generated to extremely high standards. This massively reduces the numbers of mice needed to test new compounds that we want to use in our studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We continue to monitor mouse usage on our projects by constant and careful consideration of the data and its appropriateness in addressing our experimental objectives. In developing our models we reassess group sizes as our experience develops and we will continuously look to use the minimal number of mice that provide robust experimental data adhering to standards of experimental reporting laid out in the Arrive 2.0 guidelines. In our publications we endeavour to fully report our data adhering to these guidelines to ensure experimental transparency and ensure that experiments do not need to be further repeated due to any ambiguities in their interpretation.

While our experimental models are all established and optimised, we will likely test new reagents in targeting pathways that might regulate T cell function. When this occurs, we will use small scale pilot experiments (n=3) to first test the novel reagent. Furthermore, through many years of active collaboration with industry, we typically acquire reagents from these collaborators (e.g. blocking antibodies for in vivo). This ensures numbers of mice used can be kept to a minimum as these items have been extensively validated previously and we adhere to the recommended dosing regimen in our pilot study, ensuring

less animals need to be used in establishing the experimental setup in our models. Furthermore, the purity of these reagents is often substantially higher than what is provided through commercial sources ensuring less experimental variability and smaller group sizes.

Over many years of supervising my own mouse colonies, I have developed extensive experience in efficient breeding strategies. A key approach in our experimental models is that through tracking endogenous antigen-specific responses, the data is highly consistent between experiments (rather than in models where TCR transgenic T cells are transferred for example). This means that data from different experiments can be pooled to reach the desired number of mice per experimental group. Therefore we use mice as they become available, rather than trying to generate large cohorts and this is a considerably more efficient means of breeding the mice with less mice generated and not 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.

In all of our experiments we use mice to study intestinal immune responses and in most experiments we only use genetically modified mice. We have selected genetically modified mouse models that enable robust testing of our experimental questions with minimum further manipulation (to reduce stress and discomfort through procedures) and minimum pain through tissue inflammation or damage. We have developed refined infection models and ways to assess the response to these infections.

For example:

To definitively test the role of specific pathways within intestinal T cells, we have developed models in which the responding T cells delete key genes involved with the process during their development - i.e. they do not require further manipulation/intervention such as injection of a compound to induce gene deletion.

We have developed experimental infection models that use either natural mouse pathogens which cause robust immune responses in the absence of extensive tissue inflammation, or attenuated infections where the side effects (pain through tissue damage and inflammation) experienced by the mice are substantially reduced. This means that we can drive the T cell responses we need to study using infections that cause very limited symptoms for the mice.

We use approaches to label cells within the intestinal tract using an endoscopic approach rather than surgical approaches used by other groups in the UK. This means that highly invasive surgery is not needed and the approach massively refined to cause at worst only temporary discomfort from having the endoscopy (done under anaesthesia).

We can track the progression of the infection through counting bacteria from the faeces, which can be easily collected without any discomfort to the mouse. This is substantially more refined than trying to monitor the progression of the response by analysing immune cell status within blood samples taken during the course of the infection.

Why can’t you use animals that are less sentient?

Mice are the least sentient animal species we can use. The only other model that could conceivably be used that are less sentient would be zebrafish and here the immune system and physiology is considerably different to that of humans and the relevance of our findings would be much less clear (and likely would need testing in another animal model before being relied upon for translational work). Furthermore, many of our experiments exploit the genetic modifications available in mice to maximise the detailed functional questions we can ask to provide critical new insight of relevance to human patients with intestinal inflammation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have extensively refined our experimental approaches over the last PPL and continue to review our methodology. We have consistently worked with staff in the animal facility to refine experiments and will continue to seek their advice on this project. We have reduced the need for surgical interventions through developing ways that label cells without the need to expose the target tissue. We have tested and then started to use infection models that can be monitored using analysis of faecal samples (which can be acquired with no harm or distress to the mice). Model refinement is an active and continual process that requires careful consideration of all stages of the experiments. This ethos is instilled within researchers in my lab.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All the researchers in my group are aware of the revised Arrive 2.0 Guidelines developed by the NC3Rs and recently published. We have consistently endeavoured to publish our research to the original ARRIVE standards and will continue to maintain this publication standard to ensure experimental transparency and reproducibility. In planning our experiments, we work to the PREPARE and LASA guidelines. This is taught to members of the lab when they arrive and actively discussed as researcher learn how to plan and then conduct in vivo experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Within our lab we continuously scrutinise the literature for experimental advances that might enable further refinement of our models, reduction in the number of mice used or ways to entirely replace in vivo experimentation. All members of the lab are actively encouraged to engage with this. We also engage with presentations and symposia organised by the animal facility and the NC3Rs and receive their emailed news updates. Both I and members of my lab have presented in 3Rs themed sessions, highlighting experimental refinements we have developed to try to benefit other research groups.

74. Viruses and viral vaccines research

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

virus, viral vaccines, developmental vaccines, immunogenicity, infectious disease

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult
Guinea pigs	adult
Ferrets	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To study viruses of consequence to public health to better understand their biology, natural immunity to the viruses, surveillance and health control mechanisms. The information informs the development and refinement of vaccines against viral diseases, enhances our understanding of the immunogenicity induced by vaccines, allows us to develop reagents and tests that can assess the biological quality, safety and stability of vaccines, including the vaccine viruses, vaccine formulations and viral vectors that are used in their manufacture.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the UK alone, an individual will be offered circa 11 individual vaccines to afford them with protection against 8 viral infectious diseases by the time they are an adult, more if they are immunocompromised. The adult vaccination programme adds further protection, the annual influenza vaccination campaign is designed to minimise serious illness from this underestimated disease, and travel vaccines can prevent serious mortality or morbidity as a result of contact with viruses such as Yellow fever virus, Dengue virus and Japanese Encephalitis virus. While many of these diseases seem inconsequential in modern times, influenza is the cause of 1000s of deaths annually in the UK and it is not so long ago that measles and polio caused significant mortality in this country - elsewhere in the world, these and other viral infections remain significant threats to human health, particularly in vulnerable populations such as neonates, the elderly and those living in impoverished situations.

In 2020, the WHO declared a pandemic resulting from the SARS-CoV-2 virus which causes COVID-19. This is the third pandemic of this century arising from a Coronavirus; there have been significant outbreaks of Ebola, Ziika and influenza.

Vaccines are a proven defence against infectious disease. The research and development of vaccines requires significant understanding of the natural biology of the virus causing the disease, such that vaccines are fit for purpose. It is essential that the quality of vaccines that will be used in healthy individuals and patients can be assessed by accurate and sensitive assays for specific properties - immunogenicity, safety, activity, biological quality et cetera. The work under this Project Licence will allow us to assess candidate vaccines for these properties.

What outputs do you think you will see at the end of this project?

Scientists at the Establishment participate in scientific conferences, meetings of scientific consortia etc, often including key international establishments and institutions. Here, data are discussed such that knowledge sharing can be accelerated, resulting in scientific advances in vaccine development and evaluation, with a view to bringing these nearer to licensed use in humans.

We expect to publish scientific papers in open access journals (i.e. available without the reader needing to pay) describing some of the studies that have been undertaken and the ensuing results. This dissemination of results can also help to avoid unnecessary duplicate testing using animals in other establishments.

Who or what will benefit from these outputs, and how?

Ultimately, the benefits of the work in this licence is any individual in the world who receives a vaccine against a viral disease. Such vaccines will have gone through extensive research and development phases. The work undertaken at the Establishment is critical to the collective knowledge of the potential utility, immunogenicity and safety of candidate vaccines.

How will you look to maximise the outputs of this work?

Scientists work collaboratively both nationally and internationally, which can incorporate research supported by funding bodies. Information is shared through these networks of scientific research as well as at scientific conferences.

Whenever possible, scientists publish data in scientific journals - these are 'Open Access' so that anyone can access them free of charge online. Key outputs are made available on the establishment's public-facing website.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 6000
- Rabbits: 100
- Ferrets: 550
- Guinea pigs: 20

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are tractable models for understanding key aspects of an immune response to in- development vaccines. Guinea pigs are small animal models for production of antiserum. Rabbits are a well-recognised model for the production of a broad ("polyclonal") immune response and with a greater body size, it is possible to generate volumes of antiserum that would require multiple rodents.

Ferrets are among the few animals other than primates whose response to infection with influenza reflects that of humans; both the immune response and the clinical signs closely resemble that seen in humans.

Animals in which immune responses are required are selected as adults since very young animals may have an immature, and therefore incomplete, immune response to the virus or other immunogen - this would possibly lead to a sub-optimal product.

Typically, what will be done to an animal used in your project?

The studies require inoculations and boosters of antigenic material (including but not limited to virus, virus components, nucleic acid for example) with or without adjuvant into animals using a range of administration routes including but not limited to oral, intramuscular, intraperitoneal, intradermal, transdermal, intranasal or subcutaneous. The most appropriate route would be selected based on the nature of the antigen. It may be necessary to determine the most appropriate delivery route to induce the optimum immune response from a new candidate vaccine.

Animals may have a maximum of 2 boosters (total of 3 immunisations), typically at weekly intervals;

Ferrets and mice may be challenged with influenza viruses, typically lasting 3 weeks, to assess the protective nature of candidate vaccine materials.

Blood collection will be undertaken, typically weekly.

Nasal washes, oral swabs and ocular swabs may be taken to assess antibody secretions, typically weekly.

Immunogenicity studies will typically be no longer than 6 months in duration; challenge studies may be shorter in duration.

Administration of antivirals orally to ferrets to ameliorate infection and symptoms due to infection by virulent influenza, and to reduce the likelihood of disease progression to the severity limit. Delivery will be twice daily for 7 days. The experiment will not typically exceed 3 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Experience with the procedures in this Licence has been gained over previous 5-year iterations of the work at the Establishment.

General:

Typically the majority of animals used are not expected to experience significant ill-effects.

For any animal being immunised there may be some local irritation at the site of inoculations particularly where adjuvants are used (adjuvants are chemical compounds that increase the immune response to an inoculation). Exudative skin lesions at the site of inoculation are very rare, are expected to respond to local treatments and would be expected to improve within 24 hours: if the status does not show signs of improvement with minor interventions such as those advised by the NACWO and/or NVS,within 24 hours the animal will be culled.

Blood sampling procedures are expected to cause no more than minor transient discomfort. Very rarely haemorrhage, bruising, or haematomas may occur – the incidence of these will be minimised through good technique, use of needles of appropriate size and by applying continuous gentle pressure to the site after sampling. Single-use needles are employed. Maximum blood draw volumes will be adhered to and are based on Laboratory Animal Science Association (LASA) guidelines with expert input from NVS and/or NACWO.

In the unexpected / rare case where any animal is unable to readily feed or drink as a result of their condition will be killed immediately. Any animal with deviation from normal health or behaviour which can’t be alleviated with minor interventions such as those advised by the NACWO and/or NVS, within 24 hours will be culled.

Any animal displaying neurological clinical signs, which may include symptoms such as ataxia or seizures will be culled immediately: this is expected to affect Nil to a minority of animal only.

<u>Mice, Ferrets:</u>

The adverse effects of influenza include rhinitis, fever and lethargy. Animals infected with virulent influenza viruses such as potential pandemic avian strains will usually develop

fever, loss of appetite and reduced activity levels. Sneezing, nasal discharge, partially obstructed breathing due to mucous production and diarrhoea may also occur, as can be observed during human illness. Piloerection may be seen in mice. Anti-viral drugs will be administered to ameliorate infection and symptoms. The virulence for ferrets of these viruses is variable and unpredictable, but if any symptomatic state persists for more than 24 hours, or an animal develops further signs of ill-health, action will be taken to minimise the adverse effects and suffering of the animal. There may be weight loss: a loss of up to 10% (Mild Protocol) or 15% (Moderate Protocol) which does not appear to be resolving (possibly by making available different food stuffs to encourage eating) will result in the animal being terminated. While analgesic and anti-virals may be used, if humane end points are reached, the animal will be terminated to ensure no further suffering.

Rabbits:

The use of a specific adjuvant in rabbits has been reported by this Establishment and informally by others to be associated with the risk of developing granulomas approximately 1 week post inoculation. To minimise this risk, the maximum number of inoculation sites for rabbits has been increased from 2 to 4 to minimise the inoculum volume at any one site. The areas of inoculation are depilated by clipping to improve accuracy of inoculation. Attention is paid to delivering inoculum with no seepage from the needle prior to inserting needle and on removal, thereby minimising irritation to the skin.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The overall severity under this licence ranges from Mild (Protocol 1) to Moderate (Protocol 2, 3).

For the animals that will be infected with virulent influenza, the severity may be moderate. Since the outcome of these infections can be unpredictable the animals will be monitored very closely for signs of, reduced food intake, reduced water intake, lethargy, and will be treated with antiviral medication to limit the symptoms they experience due to the infection, with particular review of the control groups (where used).

Protocol 1 will be the most applied Protocol: this incurs the least clinical impact (Mild) thus total number of animals expected to experience moderate severity is likely to be far less than the maximum indicated for the PPL.

Mice - Mild or Moderate (approx. 60:40 ratio)
Rats - Mild
Guinea pigs – Mild
Rabbits - Mild
Ferrets - Mild or Moderate (approx. 65:35 ratio).

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 data to be achieved concern the immune response and, in some cases, the protective immune response to viruses and vaccines and the pathogenesis of disease. The components of the immune response are complex and need to have a broad activity against the viruses and vaccines candidates that can mimic what might be observed in higher vertebrates - humans. Because of the complexity of the vertebrate species immune system, these can only be generated in protected animals. The same biological complexity and quality cannot be achieved in a non-animal system currently. Should the immune response to a specific antigen/antigen mix be fully characterised it might be possible to mimic this with monoclonal antibody cocktails – this will require significant research, with consideration of scientific data published in the literature, to ensure the in vitro approach is representative of the in vivo one for each antigen combination. While this is not the focus of this PPL, this would provide information that could support in vitro methods for future regulatory approaches for approved vaccines.

In some cases, data will be generated to validate in vitro assays with a future view to eliminating the use of protected species.

Which non-animal alternatives did you consider for use in this project?

Since the project licence covers activities to better understand the immunogenicity of vaccine candidates, the use of animals cannot be avoided. However, the generation of antibodies in species such as guinea pigs and rabbits will allow us to generate materials that can be produced in volumes sufficient for preparation and storage, such that they do not need to be produced fresh for each experiment.

The establishment is engaged in research into developing antibodies and nanobodies in vitro, e.g. by phage display, which would ultimately be able to replace the use of animals. However, it would be necessary to first understand the natural immune response before it can be reliably replicated in vitro. Equally, the establishment reviews the scientific literature and scientific collaborators etc should advances be made in other laboratories that can influence the animal studies and reduce the requirements for animals.

The use of cells to produce the candidate vaccine that is then used to immunise an animal removes the need for propagation of the material through an in vivo system.

Why were they not suitable?

As above, since the project licence covers activities to better understand the immunogenicity of vaccine candidates, the use of animals cannot be avoided since a fully-functional, complete immune system is required.

The elucidation of the broad immune activity of antibodies is not predictable, thus for the different novel, in-development, vaccine / virus preparations under consideration, challenging a complete vertebrate immune system will yield the necessary polyclonal response that would mimic that raised by a human if infected or vaccinated. Understanding fully the immune response in an animal is required to be able to develop in vitro alternatives (using methods such as that above) that simulate the response needed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The work under this project licence has been undertaken for many years. The numbers reflect previous years' use and allows for some flexibility should additional work need to be undertaken.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The numbers are based on previous experience and for the most part are unchanged since similar testing requirements are anticipated over the next 5 years.

During the course of the previous iteration of work covered by this PPL, the number of rats was adjusted given the increase in testing required for a range of enterovirus candidate vaccines. This is reflected in the absolute number of tests undertaken whereas the group size remained the same. This represented an increase in the overall number which has been reflected in this current PPL.

Statistical advice is available in-house to optimise appropriate group sizes based on the proposed study scientific outputs.

WHO guidelines indicate the numbers of ferrets to be used to test the safety of pandemic vaccine viruses and we can refer to this for establishing the studies under this PPL.

Higher inoculum volumes used to infect mice and ferrets with influenza viruses produce more reproducible clinical disease. Use of higher inoculation volumes will reduce the numbers of animals required for challenge studies with influenza virus. Maximum volumes will only be used where virus pathogenesis and disease are critical to the study design.

We will use archived materials, such as antiserum, from previous experiments and as far as possible these will be used in place of new animal studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The numbers of animals used under the Protocols will be the minimum required to obtain sufficient biological materials and data as based on historical scientific data and experience with the species.

The individual project plans will include a pre-study meeting to identify the number of animals required per group, in cases where the experiment has not been previously undertaken. In-house statistical expertise is available for this purpose.

Where we may undertake collaborative or contract work, we maintain dialogue with vaccine developers to determine if (and when) they can use less volume of sera to test their products, and if so we will be able to reduce the amount we provide and thus reduce the number of animals in any one study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents (mice, rats) - these animals typically will be used for the majority of the studies to investigate immunogenicity of candidate vaccine viruses. These are the least sentient animals for this purpose and are well-established in the literature for their suitability.

Rodents (guinea pigs) - generation of antiserum; Guinea pigs will be anaesthetised and bled by an appropriate blood vessel prior to and/or following immunisation: they will be bled on not more than three occasions with typically 2 weeks between bleeds.

Mice and Rats are housed in socially compatible groups. Guinea pigs will be housed in socially compatible groups whenever possible and always in floor pens.

Rabbits - generation of antiserum. Experience on an earlier licence has refined the inoculation of rabbits and thus exudative skin lesions at the site of inoculation are not expected.

We have refined the method of delivery of adjuvanted antigen during the course of a parallel and current PPL to minimise the risk of granuloma. The use of a specific adjuvant in rabbits has been reported by this Establishment and informally by others to be associated with the risk of developing granulomas approximately 1 week post inoculation. To minimise this risk, the number of inoculation sites for rabbits has been increased from 2 to 4 to minimise the inoculum volume at any one site. The areas of inoculation are depilated by shaving to improve accuracy of inoculation. Attention is paid to delivering inoculum with no seepage from the needle prior to inserting needle and on removal, thereby minimising irritation to the skin. By adapting the inoculation process the likelihood of granuloma is reduced and thus the potential for lasting harm is likewise minimised. Should granuloma form, it is possible to pause the protocol until the animal recovers sufficiently, and then select an alternate site for inoculation if needed.

Rabbits will be housed in socially compatible groups whenever possible and always in floor pens.

Ferrets - infection with influenza viruses and generation of small volumes of antiserum. In order for the ferrets to seroconvert they must be actually infected with the influenza virus, which can lead to clinical signs, and be kept alive long enough for antibodies to be produced by B cells. For animals infected with virulent influenza we will use antivirals to

reduce the clinical effects of infection: animals can be given antivirals by the oral route twice daily for 7 days beginning 2 days following an initial intranasal administration of antigenic material. It is recognised that group housing is preferable for optimum well-being of ferrets and wherever possible they will be group housed. Where possible, the use of remote measurement of body temperature will minimise the amount of animal handling required and thus keep stress levels to a minimum.

For mice and ferrets that will be infected with influenza viruses: a clinical scoring spreadsheet is in use to best monitor the clinical severity of any one animal such that interventions can be made to minimise pain, suffering, distress or lasting harm. These might include access to more palatable foodstuffs where weight loss is increasing, or the application of analgesics to ease the effect of symptoms (see below). For all animals: analgesics will be administered where needed and where their effect will not interfere with the scientific outcome of the study.

Throughout, experiments will be kept to the minimum duration to gather meaningful scientific data.

Why can't you use animals that are less sentient?

Mice and Rats are well-established in the literature for their suitability as infection models to assess immunogenicity of antigens / candidate vaccines. A mammalian model is required to present a complete immune system response to an antigen. An immature response (i.e. from a more immature development stage) may not reflect fully the requirements for a vaccine.

Ferrets are among the few animals other than primates whose response to infection with influenza reflects that of humans: both the immune response and the clinical signs closely resemble that seen in humans. Methods for more frequent observation of clinical signs have been developed for earlier recognition of onset of disease allowing earlier intervention with the use of medication to relieve symptoms or termination as appropriate. Data accumulated over the years indicate that the specific immune response is highly predictive for when a strain is sufficiently different from those that have gone before to warrant a strain change in the vaccine. The relationship is far less clear-cut for other species such as mice where infection is usually more restricted.
Rabbits are suitable for large volumes of polyclonal antisera. To generate the same amount of serum in rodents would require substantially more animals.

Guinea pigs will be used where only small amounts of serum are required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals are habituated prior to commencement of study, typically for 7-10 days.

Inoculation volumes are based on those recommended by LASA and others; they are the minimum required at any administration site. These have been shown to be appropriate over the previous licence.

We have made changes to practices under the previous PPL such as refinement of inoculation of rabbits to minimise the risk of granuloma formation. The number of inoculation sites for rabbits has been increased from 2 to 4 to minimise the inoculum volume at any one site. The areas of inoculation are depilated by shaving to improve

accuracy of inoculation. Attention is paid to delivering inoculum with no seepage from the needle prior to inserting needle and on removal, thereby minimising irritation to the skin. Reinoculation (boost) of rabbits that do develop granuloma can be paused until the animal is considered recovered.

Haematomas can occur following withdrawal of blood. This has led to routine use of needles of a shorter length and smaller gauge reducing the risk of bleeding without compromising the quality of the sample.

We have a programme of continual improvement to animal housing, husbandry and handling aimed at minimising stress in animals and encouraging natural behaviours.

Animals undergo thorough pre- and during-study checks. Analgesics will be administered where needed and where their effect will not interfere with the scientific outcome of the study. Clinical signs can be ameliorated by the use of antivirals (ferrets) or analgesia (ferrets, rabbits, rodents), for example.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance is available through Home Office including newsletters via the ASRU.

LASA and FELASA guidelines will be applied to guide dose volume/route per laboratory animal subject and surgical procedures.

NC3Rs documents on husbandry, housing and procedural work will be met or exceeded.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Establishment's AWERB circulates up to date information on the 3Rs through the Named Information Officer (NIO). Similarly, the licence applicant is on the email circulation list for the NC3Rs bulletins.
The PPL holder will work closely with the NACWOs and NVS to ensure that changes to the animal studies are improved wherever possible. This may be reflected in changes to husbandry and animal handling practices, refined procedural activities, and advances reported in the scientific literature or at scientific conferences/meetings that could result in use of a less sentient species, fewer animals in experimental groups, or non-animal alternatives.

Information exchange between PPL holders at the Establishment, in concert with the animal facility staff at the Establishment, will be used to refine the work programmes as necessary.

75. Efficacy of veterinary medicines, biologicals, feedstuffs, feed additives, residues and vaccines in the target species, including first use.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Efficacy, Antimicrobial, Vaccines, Infection, Parasiticides

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult
Pigs	juvenile, adult
Domestic fowl (Gallus gallus domesticus)	adult, juvenile
Domestic poultry	juvenile, adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to assess the efficacy of compounds that may prevent animal diseases from developing or progressing. There is a constant need to combat the development of drug resistance by parasites and microorganisms. Novel and existing compounds need to be developed, tested for efficacy and brought to market and evaluated appropriately eg. against current field strains to ensure that they are effective.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 conduct animal studies looking at the efficacy of a new or existing medicine, biological, feed additive, feedstuff or vaccine to ensure they are effective against established field strains of microorganisms and parasites. Efficacy tests are a crucial step for new or repurposed pharmaceutical and other products on their journey to market, including being approved by the appropriate regulating authority.

What outputs do you think you will see at the end of this project?

Multiple scientific reports for the sponsoring organisation that will potentially lead to new treatments for animal diseases, illnesses and conditions that cause economic and animal losses each year. Our continuing input into fundamental animal health research and scientific papers is published in high impact journals. We also play an active role in maintaining and updating guidance on animal health and wellbeing and best practise for these types of efficacy studies.

Who or what will benefit from these outputs, and how?

Animals, pharmaceutical and other companies, farmers and the wider economy will all benefit from the research carried out by our company. The testing facilities provided may result in new and novel products being brought to market to help alleviate many different animal diseases and conditions. Therefore, this benefits the animal’s wellbeing and health, farmers see improved yields and less expense for veterinary treatment and pharmaceutical companies see profits by marketing their product. These factors combined stimulate and help the wider economy to grow within the UK.

How will you look to maximise the outputs of this work?

We will work with companies, institutes and charities to disseminate the study findings, publish research papers, attend relevant conferences and play an active role in creating guidelines in this area of research.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 4000
- Other birds: No answer provided
- Sheep: 200
- Cattle: 300
- Pigs: 125

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Many of our efficacy studies are conducted in the target species for authorisation and/or support of a veterinary medicine, biological, residue, feed additive, feedstuff or vaccine and such testing is required by the regulatory authorities. There is an internal policy of checking whether non-animal alternatives are possible, for example by regular visits to the NC3Rs website (http://www.nc3rs.org.uk) and attending relevant conferences. Where there is a validated and appropriate in vitro model then the client would be informed and the in-vivo work refused unless there is a specific exemption and it is agreed with the Home Office.

Typically, what will be done to an animal used in your project?

Administration of substances / vaccine via; oral gavage, inclusion in the diet/drinking water, injections (either subcutaneous, intramuscular or intravenous), topically to the skin (e.g. plunge dipping, spraying, impregnated ear tags, collars or similar devices), implanted subcutaneously, by ear piercing e.g. an ear tag, intranasally or intramammary (via the teat canal).

Animals may be singly housed and veterinary or husbandry treatments may be withheld or varied.

The target animal may be challenged with infective parasite larvae, protozoa, bacteria or virus particles which may be administered by; mouth, nasally using a pipette (or similar device), topically to the skin (e.g. spray or pour on or by skin scrape), the mammary gland (via the teat canal), exposure to infectious agents in the environment, intravenous injection of blood borne pathogens, exposure via insertion in the cloaca. Probes may also be placed in the vagina and/or rectum.

Animals may be dried off.

Animals may have blood samples taken pre and post mortem with the possibility of cannulas being inserted into superficial blood vessels.

Percutaneous secretion sampling by needle aspiration from the dry and lactating mammary gland to allow aseptic sample collection avoiding the streak canal. This will be used once in the last 24 hours of a study. Faeces may be collected by manual removal from the rectum or sample collection from the cloaca.

Environmental temperature may be varied (protocol 7 only).

Feed may be withdrawn up to 24 hours before post mortem. Biopsies or scrapes of superficial tissues may occur.

What are the expected impacts and/or adverse effects for the animals during your project?

Likely adverse effects:

Veterinary medicines:

There may be signs of local or systemic treatment intolerance. These include:

local reactions either injection site reactions or site of application reactions (including signs of pain associated with the area, swelling, skin changes)

systemic reactions to the substance (behavioural changes, mobility changes, feed intake and weight gain changes, organ system changes)

The volume of blood removed could result in anaemia or systemic signs. Guidance table will be used to prevent this.

Vaccines:

There may be signs of local or systemic treatment intolerance particularly where multiples of normal or proposed vaccination dose rates are used during target animal efficacy studies.

These include:

local reactions either injection site reactions or site of application reactions (including signs of pain associated with the area, swelling, skin changes)

systemic reactions to the substance (behavioural changes, mobility changes, feed intake and weight gain changes, organ system changes)

Vaccination could result in an anaphylactic reaction. Artificial Infection:
The levels of infection chosen should not cause clinical disease of beyond moderate severity.

the severity limit could reach the upper border of moderate e.g. in sheep when conducting ectoparasite studies e.g. with Lucilia sericata.

normally, mastitis-causing bacteria will be chosen that would not be expected to cause systemic disease, however the upper border of moderate could be reached.

infection levels could build up e.g. on pasture during a grazing season, depending on climate and other factors.

Withdrawal of feed could result in stress in individual animals.

Increased stocking density (only birds) potential adverse effects are:

Heat stress

Pododermatitis

Lameness

Increase in sparring behaviour in cockerels Necrotic enteritis (Clostridium perfringens)
Severe depression

Diarrhoea

Dehydration

Decrease in feed consumption
Ruffled feathers

Reluctance to move Salmonella spp.

Diarrhoea (often white and watery)

Dehydration

Decrease in feed consumption

Decrease in egg production

Reluctance to move Campylobacter spp.
Up to 15% mortality rate

Decreased egg production

Rapid death due to hepatic necrosis Bacterial enteritis
Diarrhoea

Stable feed intake in growing birds

Depression

Ruffled feathers

Huddling

Sudden increase in death rate

Likely incidence:

The compounds may have undergone preclinical studies in rats or mice but these may be the first studies in the target species. It is expected therefore that, on occasion, there may be either transient clinical signs or mild lasting clinical signs that may reach the moderate severity category. For example, injection site swelling or tissue changes and swelling.

Repeated blood sampling could result in clinical signs after either a large volume is taken on a single occasion or repeated sampling over a period of time. This is very unlikely to occur as the guidance in the table within this document will be followed.

Anaphylactic reaction should be a rare result of vaccination with a product that is intended for commercialisation and would not be expected to occur in these studies.

Measures to prevent, recognise and control occurrence and severity:

prior knowledge will be reviewed to ensure that doses and dose-rate multiples are unlikely to exceed moderate severity. If they are deemed likely to exceed moderate, the work will be declined or the work will be discussed with the Home Office Inspector

the individual study protocol will include adverse events that might be seen with the substance or substances together with an assessment of their severity and a cut off limit of moderate

Animals will be closely monitored throughout the study to ensure that there are no adverse effects and should these occur they will be monitored and veterinary advice and treatment sought or the animal euthanased if the upper border of moderate severity is reached. Monitoring will focus particularly on periods when disease is likely to appear and directly after administration of test substance or challenge. Typically hourly for the first 4 – 6 hours after treatment, then at 24 and 48 hours.

Close clinical observation will be particularly necessary in key stages of some infections e.g. in fly strike to ensure removal of larvae before the 2nd instar stage of development. Similarly close clinical observations will be necessary when challenging with e.g. Staphylococcus aureus or other mastitis inducing bacteria.

should any observations approach the predetermined cut offs then a veterinary surgeon or NVS will be consulted and the animal euthanased, treated and/or removed from the study as appropriate

anaphylaxis is a rare but possible event. To handle this veterinary assistance, treatment and means of euthanasia will be available in the hours after administration

dose volumes and blood sample volumes will follow guidance

Literature review will focus not only on systemic signs, but also specific organs affected so that potential symptoms of any organ related issues can be observed as early as possible.

If possible, in a first use situation, a ladder approach will be used whereby, one animal will be infected and/or treated/vaccinated and assessments will be made on this animal before moving onto the same procedure with the next series of animals. In this way, data from a complete group will be used but it minimises the risk of a group of animals all suffering adverse effects if there are any.

Where appropriate pilot studies will be made to define scientifically valid criteria including early markers of developing severity so that these can be defined.

If an animal approaches the upper limit of moderate severity it will be treated or euthanased, as appropriate. Others in the herd or flock will be monitored and appropriately treated, removed from the study or euthanased as necessary.

Removal of feed will be assessed for scientific need against animal health and welfare. Where deemed necessary the impact will be minimised e.g. by withdrawal of feed gradually over several days with a maximum time with no feed of 24 hours.

Increased stocking density (only birds) potential adverse effects are:

Heat stress

Pododermatitis

Lameness

Increase in sparring behaviour in cockerels Increased stocking density (only birds):
Litter is checked daily and new litter added as necessary throughout the course of a study

At body weight checks, foot pad score checked

Staff are used to regularly checking birds for lameness and assessing the severity of this

Staff are used to observing this behaviour and any signs of injury will be treated appropriately. Isolation facilities will be available.

Foot pad scoring as follows (expect no more than 50% of birds to have a score of 1 for mild severity): Score Description
No lesions; no or very small superficial lesions, slight discolouration on a limited area of the footpad, mild hyperkeratosis (thickening of the outer layer of the skin) or healed lesion.

Mild lesion; discolouration of the footpad, superficial lesions, dark papillae and hyperkeratosis.

Severe lesion; epidermis is affected, ulcers or scabs, signs of haemorrhages or swollen footpads The studies are typically of short duration (1 – 3 months typically)

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 40% moderate. (see full breakdown in protocols)

Animal type	Est. numbers (mild)	Est. numbers (moderate)	Life stages
Cattle	180	120	Adult and Juvenile
Sheep	120	80	Adult and Juvenile
Pigs	75	50	Adult and Juvenile
Farmed birds	5000	3350	Adult and Juvenile

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Veterinary medicines, biologicals, feedstuffs, feed additives and vaccines must be trialled in the target species for initial efficacy purposes before taking forwards to larger trials. Unless otherwise recommended or if there is an established and validated in vitro model available, the target animal will need to be used in these studies.

Which non-animal alternatives did you consider for use in this project?

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?

We use a combination of tools to estimate the numbers of animals needed. This normally includes the NC3Rs Experimental Design Assistant, Veterinary International Conference on Harmonization (VICH) and World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines as well as consulting guidelines set by regulatory bodies and any relevant literature to ensure that the minimum number of animals is used.

Peer-reviewed journals and advice from external peers together with in-house experience and historical data over a number of years is used to ensure that animal numbers are adequate.

We are fully committed to reduction, nonetheless the exact numbers of animals required will vary with the particular study needs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have many years of experience of working with veterinary medicines, biologicals, feedstuffs, feed additives, residues and vaccines in the target species. This ensures the minimum number of animals are being used for any particular study. Due to our experience we have highly optimised our processes which enables us to maintain high welfare standards whilst delivering our clients requests. We also utilise the NC3Rs Experimental Design Assistant, VICH and WAAVP guidelines as well as consulting guidelines set by regulatory bodies and any relevant literature.

Studies are normally negatively or positively controlled, parallel studies.

Expert statisticians will be involved in study design as well as consulting guidelines set by regulatory bodies and any relevant literature to ensure that the minimum number of animals is used that are needed for a valid statistical result.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate pilot studies are used to establish an appropriate and consistent infection level that will ensure meaningful results are obtained. Dependent on the type of organism, there may be very well established in-house models which can easily produce consistent results. However some organisms respond differently to the host immune system which then can lead to unpredictable models, which may require varying the infection dose levels, the use of immunosuppression or different methods of infection e.g. in feed, water or by oral gavage. Some parasitic organisms (Ascaris suum) for example, need larger numbers of animals for successful infection, therefore a larger control cohort is required to compensate for the over dispersion of the parasite. For every model; age, immunological status, breed and strain of animal may affect the outcome of the infection negatively or positively.

Studies may be exploratory and use small numbers in a standardised design, enrolling further animals one at a time as it is established that the adverse reactions to the drug are mild or moderate and not severe, therefore slowly building up the data to a full set.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 species of animals used are the intended target species for each veterinary medicine, biological, residue, feed or water additive, feedstuff or vaccine. Regulatory bodies require efficacy data from the target animal species. To satisfy the EMEA CVMP/VICH GL 7 efficacy criteria, an overall effectiveness of over 90% is required. The species involved are: cattle, sheep, pigs, domestic poultry and other farmed avian species (e.g. ducks, pheasants, geese).

Challenge models are designed to minimise severity as far as possible whilst achieving meaningful results. The numbers chosen will normally be within the guidelines set by the appropriate guideline e.g. VICH GL12.

In some cases, naturally infected animals may be brought in for testing veterinary medicines or biologicals. Under good husbandry circumstances these animals would be given appropriate commercially available treatments to control infections. However, for scientific reasons, this is withheld in both control groups or prior to test treatments.

If the study involved mastitis causing bacteria strains would be chosen that would not be expected to cause systemic disease.

We will seek to link physiological criteria with behavioural signs in a heat stress model.

Why can’t you use animals that are less sentient?

We have to use these animals as hosts due to the research being directly related and utilised in real world farm animal and bird environments. However we regularly refine and/or validate existing animal models in support of 3Rs initiatives

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We minimise suffering by examining each study plan to identify opportunities to improve welfare by, for example, using experienced and knowledgeable staff, handling and habituating animals to procedures where necessary, meeting or exceeding codes of practice and current guidance or literature suggestions for enrichment.

The animals used are the target species for the veterinary product on trial. Any models will have welfare at the centre of their design. In addition, animals on these studies will have a heightened level of observations immediately after and in the days following administration of any substance. Our standard level of monitoring is a minimum of twice daily. Frequency of observations are increased on an as needed basis based on animal observations and behaviour. For example, observation frequency could increase to hourly monitoring following the appearance of clinical signs associated with mastitis.
Observations would normally be general health observations but these are supplemented with clinical observations by our NVS (or similar), monitoring of temperature and other parameters as necessary.

If a treatment is to be administered over the course of a number of days, such as in feed or water, then we would aim to administer at the same time daily to allow the animal to become accustomed to the dosing regimen. We may also associate dosing and administration with feeding times as positive reinforcement for the animal If it is believed an animal is approaching and likely to breach the predetermined severity category then we have a well-established process. This includes seeking veterinary advice from our NVS or another veterinary surgeon, discussions with the NACWOs, PELh, PPLh, PILh and Study Investigator. This group of people decide the best next steps in terms of animal welfare. This could be either, immediate alleviating of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, and increased monitoring if the animal is unlikely to breach the severity limit by the conclusion of the study if appropriate.
If an animal breaches the predetermined severity category for the study then immediate action is taken. This often includes rapid discussions with the NVS, PELh, PPLh and PILh alongside the study investigator. We would also if possible contact an ASRU inspector. The course of action could be immediate alleviating of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, maintaining and closely monitoring the animal if the scientific need is justified and the end of the study is within a short time frame, if agreed by an ASRU inspector. All decisions are taken on a study by study basis and differ with treatments and species.

We will also continually review our procedures and refine as much as possible with guidance from, for example, VICH, EMA, WAAVP and other relevant organisations and published literature.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

WAAVP guidelines for best practice in parasiticide related work. These guidelines are now seen by most regulatory authorities as the standard by which parasite-related protocols are written, and consider numbers of parasites required for study purposes. Similar guidelines will be utilised for bacteriological, virological and other types of study.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly reference the NC3Rs website, published literature, attend relevant conferences and employees are members of The Royal Society of Biology and other relevant organisations.

76. Investigation of Thymus Degeneration and Regeneration

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

T cells, Thymus, Immunity, Repair, Atrophy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project is to study the cellular and molecular mechanisms that result in the loss and regeneration of functional thymus tissue and T cell development following damage or disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The thymus is the primary organ responsible for supporting T cell development. T cells form an essential component of the immune system, providing protection against both infection and tumour formation. In addition to supporting the development of T cells that protect against infectious challenges, the thymus also limits the development of T cells that may otherwise drive autoimmune disease (a process termed central tolerance). Continuous production of new T cells is important to maintain effective immune protection by replacing T cells that can be gradually lost from the peripheral circulation. However, thymus activity is not constant throughout life with a loss of thymus tissue (termed thymus

atrophy) occurring in response to numerous challenges including: acute damage (e.g. irradiation exposure), obesity and therapies used for treatment of blood cancers such as high dose irradiation. Therefore, while the thymus is critical for the recovery of T cell immunity following such challenges, the sensitivity of the thymus to induced atrophy can create severe immunodeficiencies, for example dramatically heightened susceptibility to life-threatening infections following treatment for blood cancer. Further, alterations in the ability of the thymus to control the development of autoreactive T cells may also lead to the development of autoimmune disease following thymic atrophy. Interestingly, the thymus possesses an inbuilt regenerative capacity to counter such effects, importantly however the mechanisms that control both thymus degeneration and regeneration, including how this impacts the development of functional, self-tolerant T cells are very poorly understood. In addition, although different challenges such as low or high dose irradiation and obesity all lead to thymus atrophy, the mechanisms involved in each appear to include unique elements, including how and the degree to which the thymus is able to recover following degeneration. These limitations therefore hinder the creation of new approaches to improve thymus recovery and T cell driven immunity. This basic science project aims to investigate the cellular and molecular networks in the thymus that control recovery after induced atrophy and examine whether such mechanisms are either shared or unique to the different atrophy-inducing stimuli of either irradiation or obesity.

What outputs do you think you will see at the end of this project?

Work performed under this licence will:

Generate basic science data that will enhance our fundamental understanding of the cellular and molecular mechanisms controlling thymus atrophy and regeneration.

Provide a platform for the identification of new pathways and targets that may inform follow-on future studies targeted towards the development of translational approaches to manipulate and enhance thymus function.

Generate scientific findings that will be communicated to the wider public and scientific community through presentation at scientific meetings, public engagement activities and publication in high impact peer-reviewed scientific articles.

Who or what will benefit from these outputs, and how?

The outputs from this will be of interest to a wide range of individuals and groups interested in understanding thymus function, and exploring the potential for manipulation of thymus tissues in order to enhance T cell development and output. For example following the use of ablative therapies used to treat patients with blood cancers that amongst other outcomes, can lead to the depletion of T cells, will be of interest to both clinical and non-clinical colleagues. It is anticipated that the immediate beneficiaries of this research will include basic research scientists within the field of biomedical research interested in T cell biology and the impact of perturbed T cell development on immune protection. At a local level, interactions with colleagues will ensure discussion and distribution of data and findings from the project. Wider beneficiaries will be engaged through sharing of information through attending and presenting data at national and international meetings. Results from the project will be published in free-to-access scientific publications.

Importantly, this is a basic science project. It is essential that such exploratory projects are undertaken if we are to identify novel mechanisms that regulate thymus function and T cell development and thereby make new discoveries that further our understanding of thymus

biology that may ultimately inform approaches to manipulate the immune system for improved health. Although outputs from the project may be of interest to the clinical community, the likely potential for any outputs to directly inform future therapeutic approaches should be viewed in the long term. In addition to individual beneficiaries, the ultimate potential long-term goal to translate research findings into methods to improve immune function in humans may include socio-economic impacts due to health and wellbeing implications of poor immune function occurring as a result of reduced thymus function.

How will you look to maximise the outputs of this work?

In order to maximise outputs from this work, we will continue to maintain existing and develop new collaborations with colleagues at both national and international levels. Such collaborative interactions are anticipated to enhance sharing of both knowledge and materials that will provide added value to both this programme of work and others. Outputs from this programme of work will be shared with the scientific community through communications of results and technical approaches at meetings and conferences e.g. by both oral and written presentation. Such dissemination of work will raise the profile of work and allow sharing of data outputs that will enhance knowledge exchange and support development of our projects via feedback from experts in the field. In addition, we will continue to publish our results in high-impact journals. Importantly, in line with the requirements of our funding bodies published results will be in open-access scientific journals in order to make our research as accessible as possible. We also aim to publish negative results where these will provide important data that may inform other studies or prevent replication of experiments elsewhere. In order to communicate outputs with wider audiences we will continue to use additional platforms, including social media to maximise exposure of results and publications. Where possible we will also engage with the wider public through public engagement events in order to communicate our work to wider audiences.

Species and numbers of animals expected to be used

- Mice: 15,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The immune system is formed by a network of cells and inter-connected organs (termed lymphoid organs) that are spread throughout the body. In this programme of work we aim to look at how the thymus, a primary lymphoid organ that supports development of T cells, undergoes degeneration and subsequent regeneration following challenges such as irradiation damage or in settings such as obesity. Importantly, T cells exported from the thymus are critical in the maintenance of immune protection against bacterial, fungal and viral infections and for the prevention of tumour formation. Therefore, loss of thymus tissue and reductions in T cell development and output from the thymus can have serious implications for immune protection throughout life.

In particular, this project aims to study thymus atrophy and recovery at adult stages of the life-course. In order to investigate these aims, this project will use mice, including genetically-altered animals as an experimental model. The mouse represents the lowest order of placental mammal that possesses an immune system of similar make-up and function that can be used to effectively model the human immune system, including the thymus. Importantly for example, one of the primary aims of this project seeks to model and study thymus atrophy and regeneration that occurs following the use of therapies used to treat human patients for blood cancers. The treatment of such disease involves exposure of patients to radio- and/or chemotherapies in order to deplete cancerous cells in the bone marrow, followed by transplantation of donor bone marrow containing stem cell populations. The use of mice therefore represents the lowest order placental, mammalian species that possess a corresponding system of lymphoid organs. In addition, mice represent the lowest order species where haematopoiesis (blood cell development) occurs within the bone marrow, and subsequently leads to a programme of T cell development and output from the thymus and can therefore can be used to model this process of thymus damage and T cell recovery in humans.

Typically, what will be done to an animal used in your project?

Under this basic science licence, we will undertake investigations to study the mechanisms that can result in both the loss of functional thymus tissue (thymic atrophy), and subsequent regeneration, including the impact of these processes on T cell development. All animals will undergo a procedure to induce thymus atrophy in order to understand the cells and molecules that drive this process. Thymus atrophy will be induced by either exposing animals to a low dose of irradiation on a single occasion, feeding a high fat diet for up to 20 weeks, or exposing animals to a higher dose of irradiation on up to two occasions accompanied by a transfer of donor haematopoietic (blood) stem cells delivered by a single intravenous injection. In order to study the role of defined cells or molecular pathways in either atrophy or regeneration, some animals may then receive a single intravenous injection of cells on one occasion, whilst other animals may receive an injection of substances such as proteins, peptides or antibodies via either intravenous or intraperitoneal injection on up to 6 occasions over a maximum period of two weeks, with injections separated by a minimum of 24 hours. In order to investigate outcomes of such procedures on thymus and T cell recovery, animals will subsequently be humanely killed, and cells/tissues isolated for post-mortem analysis.

In order to study the functionality, including self-tolerance of T cells generated in atrophied/regenerated thymus, in a small number of experiments T cells, isolated post mortem from mice having undergone induced thymus atrophy, will be transferred to host mice via a single intravenous injection. Animals will then be maintained and subsequently humanely killed , and cells/tissues isolated for post-mortem analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential adverse effects that animals may experience include:

High fat diet: Increase in bodyweight and greasy coat due to feeding animals a diet with a high fat content (typically over 8-12 weeks).

Irradiation exposure and stem cell transplantation: Animals may experience temporary weight loss (1-2 days) following exposure to irradiation. Animals may also display signs of

radiation sickness between day 6-14 post-irradiation before donor stem cells are able to reconstitute haematopoietic compartments (blood cells).

Delivery of cells OR substances e.g. via either intraperitoneal or intravenous injection: temporary stress and discomfort due to handling and insertion of needle for the duration of the injection (less than 1 minute).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate 100%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The analysis of thymus atrophy and recovery relies on the coordinated activity of diverse cell types and molecular signals that require the use of whole animal models to investigate in detail. For example, the thymus does not contain haematopoietic (blood) stem cells that develop into T cells. Rather, in the adult, blood stem cells are located in the red marrow of skeletal bones and migrate from these distant sites in the body through the bloodstream and subsequently colonise the thymus. Importantly, this is a continual process meaning that the study of T cell development relies on an interconnected system of skeletal and circulatory blood vessels with the thymus. Following development in the thymus, a further step is the export of T cells into the peripheral circulation in order to provide effective immune responses. Similar to thymus colonisation, in order to study this critical process it is necessary for the thymus to be fully connected to an intact circulatory system. In particular, under this project we aim to study how the thymus undergoes atrophy in response to challenges such as obesity and irradiation and subsequently how the thymus recovers. Importantly, thymus recovery occurs over an extended period of time (>8 weeks) and as yet no in vitro experimental systems exist that can effectively model this complex, interconnected network of circulatory, skeletal and lymphoid tissues systems including the thymus over a protracted period of time.

Which non-animal alternatives did you consider for use in this project?
We routinely use in vitro (in a dish) models in our research, including cell and thymus organ culture systems. These approaches include reaggregate thymus organ culture systems that allow for the study of thymus biology without using intact whole animal models. These models therefore help to reduce the need to perform experiments in whole animals, and provide a key system where we are able to validate experiments in vitro e.g. screening and testing the ability of defined molecular pathways to influence thymus function. Although such in vitro systems provide an invaluable approach to explore the role

of defined cells types and molecular pathways, thereby reducing the need to use experiments in animals, they are currently still limited in their application to the study of thymus degeneration and regeneration. Current limitations to such models, such as lack of a connected blood supply and therefore an absence of immature T cell recruitment from the bone marrow, currently restrict the use of such models as a complete replacement for whole animal models. A further current limitation to the use of non-animal models for thymus research remains the lack of information regarding stem cell populations that are capable of generating functional thymus tissues. Although our previous work, and that of other research groups, have sought to identify stem cell populations that can form complete thymus tissues as yet no specific markers have been identified that allow for the isolation and propagation of such cells in vitro, thereby precluding their use in alternatives to animal models.

Why were they not suitable?

The thymus contains a unique three-dimensional network of cells that support the development of T cells. At present, attempts to use cell culture models to recapitulate normal thymus structure and function, in particular use of 2-dimensional cell culture models has met with limited success. The lack of specific markers to isolate stem cells from thymus tissues in order to attempt generate thymus tissues has also hampered efforts to replace animal models. Recent efforts have also been made to utilise organoid systems to study aspects of thymus biology, although as yet the effectiveness of such systems remains limited and have failed to effectively model normal thymus development and function. Critically, the need for a fully integrated immune system that communicates and directs the migration of immune cells through a system of blood vessels is essential for the study of the thymus atrophy and recovery. As such, alternative models to study thymus function currently remain limited. However, we will continue to study the scientific literature for any emerging models that may facilitate reduction and replacement of animal models, and will continue to utilise and refine our current use of non-animal models such as thymus organ culture systems 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 programme of work is a continuation of work performed under our previous project licence. We have estimated our numbers based on our experience of animal husbandry and use of wildtype and genetically altered animals using such protocols and procedures over the last 5 years of our previous project licence. Numbers of animals are therefore based on previous Home Office data returns for the use of animal numbers, in-house data from pilot studies and ongoing/published studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design phase we use power calculations (e.g. using G* Power software) to estimate the predicted group sizes necessary to determine statistically significant results. In order to reduce animal use, we routinely use robust approaches to ensure standardisation of experiments in order to make them directly comparable for example where an individual scientist repeats an experiment across different days, or where experiments are performed by more than one individual. We adopt standard experimental approaches within our research group to ensure reproducibility via the use of shared experimental techniques and regular lab meetings to ensure that all research lab members are performing experiments in exactly the same way. We also perform regular calibration of our scientific instruments again in order to reduce the need to repeat any experiments due to variability in the acquisition and recording of results.

Wherever possible we use control animals from the same litter as experimental mice in order to both reduce unnecessary animal breeding and to reduce the potential for any observations occurring as a consequence of differences in genetic and/or age-related factors. Where undertaking a new series of experiments we will perform pilot experiments coupled with power calculations to estimate the number of animals that we need to breed, maintain and subsequently use in experiments. We will also make use of NC3Rs resources for 'Experimental Design and Reporting', including the experimental design assistant, (https://nc3rs.org.uk/experimental-design-and-reporting) to support our studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to optimise our use of animals, we will use a combination of approaches:

Efficient animal breeding and husbandry. Mouse colonies will be maintained at a size necessary to generate animals required for robust experimentation. Resources such as those provided via the NC3Rs' Breeding and Colony Management Hub will also be used to support this. Wherever possible, we will use litter-mates as control mice in order to optimise maximum use of animals generated under this licence.

Pilot studies. These will be undertaken whenever we commence a new series of experiments or use new reagents etc. Outputs from such experiments will allow us to accurately plan animal breeding and subsequent experiments for example through the use of power calculations.

Sharing of materials. We undertake regular team research meetings at the start of every week where we discuss experimental plans. Here we are able to maximise use of every animal by combining experiments and sharing tissues/cells taken from each animal.

Archiving materials. We regularly archive cells and tissues by freezing and storing materials. Such archived tissues provide a valuable resource for future experiments, for example gene expression and microscopy analysis of cells/tissues. Using such approaches we are able to maximise use of tissues and reduce the need to breed additional animals.

As described above, we will make use of NC3Rs Experimental Design and Reporting Resources to guide our studies, and will ensure experimental design and conduct is performed and reported in line with the ARRIVE 2.0 and PREPARE guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This programme of work will be supported by the use of both genetically-normal (termed wildtype) and genetically-altered mice in order to explore the cells and molecules that control thymus degeneration (atrophy) and regeneration.

The thymus undergoes a process of atrophy in response to numerous damaging stimuli such as exposure to irradiation, including: 1) low dose irradiation, 2) high dose irradiation, for example as used in ablative therapies used for treatment of haematological (blood) cancers and 3) obesity. Importantly, the response and recovery of the thymus may differ in response to these varying types of challenge. We therefore propose to study thymus atrophy using complementary models that are relevant to human conditions and that cause the least adverse effects to animals whilst addressing our scientific questions.

Irradiation models.

In this project, we will expose adult mice to two different levels of irradiation:

Low level irradiation. Here mice will be exposed to a low dose of whole-body irradiation that whilst sufficient to induce damage to highly radio-sensitive tissues of the thymus, does not lead to depletion of peripheral cell types, including stem cell populations in the bone marrow. This model has been selected as a model of acute thymus damage that is capable of driving thymus atrophy, whilst sparring overt adverse/damaging effects on other more radioresistant cells in other parts of the body. Of note, the sensitivity of irradiation exposure is linked to the genetic background of mice. In order to ensure the least possible adverse effects are experienced by any given animal, the minimum possible dose of irradiation is tailored to the genetic background of mice in each experiment performed.

High level Irradiation and stem cell transplantation. In order to model therapies used in human patients for treatment blood cancer, we will expose mice to a higher dose of whole body irradiation. This approach induces cell death in haematopoietic stem cells that generate all cells of the blood system, including T cell progenitors. All mice undergoing irradiation in this model will receive a transplant containing haematopoietic stem cells that will subsequently restore haematopoiesis (blood cell development). All animals are expected to make a full recovery following infusion of donor stem cells via a single intravenous injection.

Importantly, it is again appreciated that the genetic background of inbred mice can lead to differences in sensitivity to irradiation. We will therefore ensure that irradiation doses are tailored to the known radiosensitivity of different genetic backgrounds of mice used in this study in order to minimise the impact of adverse effects.

Diet-Induced Obesity

In order to model obesity driven impacts on thymus, we will use a diet-induced model that involves feeding mice an altered energy diet (up to 60% kcal coming from fat). The delivery of such diets to mice have not been associated with the development of physical or behaviourial abnormalities, and as such are not typically anticipated to cause pain, suffering or distress. However, the coats of animals fed diets high in fat content may become greasy and lead to excessive grooming with associated irritation skin irritation.

Why can't you use animals that are less sentient?

In this project we will use mice as an experimental model. The mouse replicates the mammalian placental pattern of human development over a gestation period of 21 days and provides the closest model that replicates the development, maintenance and loss of thymus function with increasing age. Of note, placentation in mammals is associated with the development of specific properties of the immune system that are not present in lower order species. For example, this includes the evolution of cellular and molecular mechanisms of the immune system that aid in preventing maternal rejection of the developing fetus eg regulatory T cell development in the thymus. Importantly, the mouse represents the lowest order species that contains a similar full makeup of primary and secondary lymphoid organs and tolerance mechanisms that regulate autoimmune activity that collectively replicate those observed in humans. The mouse is also the only species that provides a wide range of natural and induced mutants with defined genetic alterations that allow the study of target molecules crucial to the function of the immune system. A large body of published data has been generated characterising the immune system in murine models, and the vast majority of knowledge regarding thymus development and function is solely based on the use of murine models since the first formal description of the critical role of the thymus in T cell development in the 1960s using mice as an experimental model. Importantly, the use of species that are less sentient remains limited. For example, Drosophila fly models do not possess an equivalent of the lymphoid lineage, including T cells nor do they possess a thymus which is evolutionarily restricted to jawed vertebrates. While fish models e.g. zebrafish possess a thymus, notable differences preclude their use as a model to fully replace the use of mouse models, particularly in regard to studying thymus biology in relation to bone marrow transplantation due to these animals lacking bone marrow haematopoiesis with blood cell development taking place in the kidney of adult fish. Although this project will use embryonic models wherever possible, we will however need to extend use of animals to adult stages in order to track postnatal stages of thymus development and maintenance that include critical stage specific roles for the thymus in T cell production.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our research group has played a significant role in refining experimental approaches to study thymus development and function in mouse models. For example, we have played a major role in developing novel in vitro models for the study and manipulation of thymus tissues. Such approaches have been widely adopted within the international research field and have provided an essential model to validate candidate cellular/molecular pathways without the need to use complex in vivo models.

We will always aim to keep our experiments to the minimum possible duration to meet our experimental end-points, and will always use the fewest possible interventions (e.g. injections) that are needed. Best practice in animal husbandry e.g. refined handling techniques and provision of enrichment items will also be adhered to, and as guided by the NC3Rs'. In order to minimise welfare costs, as is required, all relevant work will be

carried out under appropriate aseptic conditions which will be of particular relevance if strains exhibit immunodeficiency following treatments such as irradition exposure. Where mice have for example undergone irradiation exposure, animals will undergo increased monitoring to ensure appropriate recovery and haematological reconstitution. In addition, where larger doses of irradiation are used to model stem cell transplantation, we will use split doses of irradiation (e.g. two separate low dose exposures) as opposed to a single high dose exposure to minimise the impact of irradiation-associated adverse effects.

Where mice are fed a high fat diet, coats of animals will be carefully monitored, and soft bedding will be used to avoid skin irritation. In addition, high fat food will be placed on the floor of the cage to avoid any pellets dropping on the coat from suspended food containers. Where any mice show signs of pain or irritation, animals may be treated using analgesics following consultation with appropriate staff NACWOS and/or the NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our experiments will be planned, conducted and reported in line with the PREPARE (planning) and ARRIVE (reporting) guidelines. We will also make use of the NC3Rs Resource hub e.g. 'Breeding and colony management' and 'Experimental Design' to inform our approaches. LASA guidelines will be adhered to and used where appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to make good use of NC3Rs resources both through the NC3Rs website, and also at a local level via communications and activities driven by a local 3Rs group e.g. regular distribution of the NC3Rs newsletter. We will seek to engage with relevant seminars, conferences and webinars where available, and will maintain contact with NACWOs, and the NVS where appropriate. As described elsewhere, we will also continue to review the scientific literature to ensure that we are able to identify and potentially adopt any new approaches and/or models that may supplement or replace our current animal models.

77. Improving treatments for brain tumours

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

Brain cancer, Glioma, Radiotherapy, Radiosensitisers, Toxicity

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to test the effects of new treatments that may improve the outcome for patients with hard to treat brain tumours, especially aggressive primary brain tumours in adults.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The most common primary brain tumours in adults are aggressive, high-grade gliomas. They remain extremely challenging to treat and do not respond to conventional treatments including surgery, radiotherapy and chemotherapy

What outputs do you think you will see at the end of this project?

Publications describing the effects of new treatments or new combination treatments that may be useful to test in patients.

New information about how different treatments may be best combined to improve how well they work. New information about how to avoid toxicity of new treatments or new combinations.

Who or what will benefit from these outputs, and how?

In the long term (5-10 years) patients with brain tumours may benefit from having access to new treatment approaches.

In the short and medium term the specialist community in brain tumour research will benefit from increasing knowledge of which new treatments may work and how they work for brain cancers.

How will you look to maximise the outputs of this work?

Effective dissemination of new knowledge through local, national and international meetings.

Working with national and international collaborators including in established consortia to bring new treatments to the clinic as quickly as possible.

Feedback to the relevant research community about challenges and unsuccessful aspects of the project.

Species and numbers of animals expected to be used

- Mice: 1200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice for these experiments as they are the smallest species that can be used in our pre- clinical imaging and treatment equipment. Mice models of glioma, especially using injection of tumour cells into brain, have been well validated as effective models of the human disease in terms of tumour and normal tissue responses. We will use adult mice for the majority of these experiments. In some cases, particularly when we are assessing the effect of treatment on normal brain we may also use younger animals to model the effect of treatment on survivorship in young patients.

Typically, what will be done to an animal used in your project?

In a typical experiment animals will be injected with tumour cells direct to the brain while under anaesthesia using a stereotactic guidance technique. Animals will then be monitored over a number of weeks (typically 3-6) until tumour growth is expected. At this point animals may be imaged, for example by MRI or bioluminescent imaging to assess tumour size. In each case animals will be under anaesthesia for the period of imaging. Animals will then be allocated to treatment groups, commonly a short course of radiotherapy (3 doses per week over 1 or 2 weeks) and/or drug treatment given by gavage or ip or tail vein injection.

Following treatment animals will be closely monitored for signs of ill- health over the subsequent weeks and may also undergo further imaging (MRI, bioluminscence) at specific time points. We have set limits for interventions so that there is at least 24 hours between imaging sessions and no more than twice per week and no more than 6 imaging sessions will be performed within any one month period. A typical experiment would last 2-3 months.

Less commonly we will implant tumour cells under the skin and monitor tumour growth directly by caliper measurement across treatment groups described above.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected effects of surgery on these animals include pain from craniotomy or subcutaneous injection and/or from problems with wound healing, which may last from hours to a few days after surgery.

The expected effects of tumour growth on these mice include poor feeding and weight loss, reduced or abnormal mobility, reduced interaction with cage mates, undergrooming. The expected duration is a few days, after which animals will be sacrificed if these signs do not resolve or worsen.

The expected effects of treatment include poor feeding, pain (from injection) and after-effects of anaesthesia including reduced mobility and low body temperature. These effects are expected to be short lived (hours).

The expected effects of other investigations include pain from injections of contrast for imaging, which is expected to be short-lived (minutes or hours)

Animals with subcutaneous tumours may also experience discomfort from tumour ulceration.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for mice are moderate in the majority of animals. Predicted severe in <1% due to exceptional circumstances

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although in vitro (2D and 3D) as well as new organoid models provide useful means to test

specific hypotheses and/or validate findings from screening experiments, in vivo models are a vital addition in clinical translation. They are the only means of assessing the impact of tumour micro-environment on response to treatment, which can often have a powerful modifying effect. In addition these models bridge the gap between biological end-points such as tumour cell killing and clinically relevant outcomes including symptom-free survival. They also permit investigation of clinically relevant biomarkers, for example new imaging approaches to monitor tumour responses which cannot be applied outside of a full organ model. In vivo work is also critical to assess the effects of treatment on normal tissue since this depends on the interplay between different cell populations that cannot be recapitulated in vitro.

For some glioma models, especially patient-derived tumour models, cells cannot be maintained in vitro so direct implantation to mouse brain is necessary to maintain them.

Which non-animal alternatives did you consider for use in this project?

We have used 3D spheroid models for initial proof of concept and would also consider tumour/normal brain organoids, which may allow us to focus the in vivo work on fewer agents and reduce animals needed for pharmacodynamic or tissue based end-points of drug/combination effects.

We are working with collaborators to introduce a combined normal brain/tumour organoid which may permit assessment of effects on tumour and normal tissue simultaneously and further reduce the numbers of mice needed for definitive experiments.

We also have links to labs developing tissue on chip or tumour on chip devices which would support efficient and relatively high throughput assessment of novel agents. We will utilise all of these models when appropriate.

Why were they not suitable?

3D spheroid models do not incorporate normal tissue components and cannot be used for clinically relevant translational end-points.

Organoid and tumour on chip technologies are still in 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?
This estimate is based on numbers used previously and the likely balance of work going forwards, which is unlikely to change significantly. I have taken in to account the reduced experimental work carried out 2020/21 during the Covid pandemic when access to in vivo work was severely restricted.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We always use models with high take rates and reproducible growth characteristics (eg the mouse tumour CT2A) unless there is a specific indication for a different model, for example due to expression of a specific target or an indication to use patient derived material. This very significantly reduces the number of animals required per treatment group (usually 5) compared to when we use patient-derived material to establish tumours (usually 12).

As far as possible we design experiments so that we can cross-refer between control/standard treatment groups, reducing the groups necessary across a series of similar experiments using the same model. We use standard radiotherapy/chemotherapy regimes across experiments to facilitate this approach.

We also use data from previous experiments, including serial tumour imaging/monitoring data to inform study time points, for example for histological assessment so that the numbers of animals included for these end-points are minimised.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We use efficient breeding in house as well as use of external suppliers to prevent animal wastage.

As above, we use models that we are familiar with and for which we have significant prior data as far as possible. For experiments using new or unfamiliar models we routinely use data from the literature to design small pilot experiments before embarking on large treatment/efficacy work to ensure efficient experimental design.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the lowest sentient animal that can be used for these experiments for clinical translation.

We use established syngeneic and patient-derived xenografts for much of this work, for which we have high quality data on tumour growth, expected symptom-free survival and treatment effects. This allows us to design experiments in which mice undergo treatment at early time points prior to tumour-induced symptoms and to monitor them closely to ensure minimal duration of tumour-induced symptoms.

We also use state of the art technology to administer treatment, including highly targeted radiotherapy, to minimise the side effects of treatment since only the tumour and small volume of normal tissue receives a high dose. We routinely design experimental protocols

to ensure that post-treatment effects can be monitored during the normal working day, for example by treating cohorts early in the day rather than spreading treatment through the day.

Whenever possible we will use imaging-based end-points (MRI, bioluminscent imaging) in place of survival/symptom based end-points to reduce animal distress and suffering.

Why can't you use animals that are less sentient?

Mice are the smallest animal species that can be use in our pre-clinical imaging and radiotherapy equipment. We need to use models that fully recapitulate the human disease as far as possible and we have selected models that reflect this. These models include a relevant tumour micro-environment and allow assessment of effects of treatment on both tumour and normal tissue.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will work with local and national groups who are developing relevant technologies for these experiments. This includes local collaborators who are working on novel imaging approaches for tumour monitoring including small animal PET.

We also work with the CRUK Rad-Net pre-clinical radiotherapy group who are developing an integrated radiotherapy-MRI imaging bed set up to allow MRI-based radiotherapy planning to further refine the accuracy of this treatment and further reduce unnecessary radiation exposure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow current guidelines including:

The updated (2020) guidance on animal testing and research.

UKCCR guidelines for the welfare and use of animals in research (British Journal of Cancer (2010) 102: 1555-1577).

LASA guiding principles for preparing and undertaking aseptic surgery

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We run a local user group for all researchers involved in in vivo work and for our husbandry and in vivo unit staff. This reports to the institution- wide committee who are responsible for in vivo work across the whole campus. New approaches to procedures/husbandry etc are discussed at these meetings. All researchers all also circulated regular updates from for example the NC3Rs unit (Replacement, Reduction and Refinement guidance).

All practitioners are encouraged to maintain/improve their skills through regular courses as well as meetings with researchers in other units.

Specifically for this project we work closely with other institutions doing similar work through radiotherapy research networks and are members of the pre-clinical drug-

radiotherapy working group. This ensures that developments in technology, in model development and in experimental approaches are shared across the UK.

78. Breeding and maintenance of genetically altered (GA) animals, re-derivation, and GA embryo/gamete storage

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Transgenic, Conditional expression, Cancer, Neurodegeneration, Re-derivation

Animal types	Life stages
Mice	adult, neonate, juvenile, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Genetically altered (GA) rodents are of great value in understanding the function of genes and pathways important in physiological and pathological processes and to produce models of human and animal disease. The purpose of this work is to breed, maintain and generate mice with defined genetic alterations and supply them or their tissue for research into the control of disease, ill-health or abnormality and/or the study of normal and abnormal physiology, biology or behaviour.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The mice produced will be used for the understanding of basic biology and the discovery and development of new medicines for the treatment and prevention of diseases. This licence will also allow us to provide GA tissue for laboratory use, store lines in a frozen state (cryopreserve) when not required and send or receive frozen embryos from academic and commercial suppliers in accordance with the principles of the 3Rs.

We currently breed approximately 100 GA breeding lines used by around 20 different research groups. A single service project allows a person with expertise on the breeding and husbandry of GA animals to be in full control of the GA breeding programme with anticipated welfare benefits and is efficient where several research programmes may use the same animal strain. The experience and expertise available in animal husbandry will ensure that breeding programmes are co-ordinated to ensure optimal animal husbandry and minimal wastage producing only animals as needed. Minimum numbers will be used to maintain colonies, further embryo cryopreservation will ensure that strains are not lost or bred unnecessarily. The scientific justification for the animal usage is provided by the receiving project licensees or by the scientists receiving tissues.

What outputs do you think you will see at the end of this project?

The output will be to efficiently produce GA mice of a high health status while minimising the number of unused animals. These will be supplied to other project licences or used to supply tissue for laboratory research within the university. The mice supplied to other PPLs or tissue produced will be used to research into the control of disease, ill-health or abnormality and/or the study of normal and abnormal physiology, biology or behaviour. This will produce novel publications giving information on the pathways controlling these processes and will be used for the discovery and development of new medicines for the treatment and prevention of human and animal disease.

The licence will also enable us to produce embryos and sperm from GA mice which can be used to cryopreserve breeding lines in house and to send and receive frozen lines from academic and commercial suppliers.

The outcomes of research using either tissue or live mice produced on this licence will be circulated through publications and presentations at conferences, and go on to inform new therapies and treatment strategies. Researchers will participate in public outreach activities to inform the public about their research and its relevance to disease.

Who or what will benefit from these outputs, and how?

Over the five years of the licence, the mice produced are expected to be used in research that will advance research in the control of disease, ill-health or abnormality and/or the study of normal and abnormal physiology, biology or behaviour, and will be used for the discovery and development of new strategies for the treatment and prevention of human diseases. This includes cancer therapeutics, neurodegenerative and metabolic disease.

How will you look to maximise the outputs of this work?

Outputs will be maximised by carefully designed breeding programmes in collaboration with the end users of the animals or tissue, combined with efficient running of the biomedical research facility

Species and numbers of animals expected to be used

- Mice: 88450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

GA animals, particularly rodents, are currently in widespread use in biological, medical, and veterinary science and have been shown to be of great value in elucidating the function of genes and pathways in a wide variety of biological, physiological, and pathological processes. The human genome has been sequenced, as have a number of other genomes (partially or completely), notably the mouse. However, the function of many genes is not known or is not fully understood, either individually or in the ways they interact to produce their intended effects, or how they are dysfunctional in disease. To better understand normal physiological processes and abnormal disease processes requires, when necessary and justified, the use of whole animal models.

Within biomedical research generally and at this institution the use of GA animals in many stages of research are well established. Their value has especially been demonstrated, for example as research models for cancer, neurological, metabolic, and inflammatory diseases and their therapy, as well as aiding fundamental research into human physiology.

Typically, what will be done to an animal used in your project?

There are two protocols (4,5) for the breeding of GA animals. Protocol 4 covers the breeding of GA strains that do not have any physical (phenotype) consequences, or where the phenotype is within the mild severity band. Protocol 5 will allow breeding where the phenotype causes adverse effects of a moderate severity. In both protocols a tissue biopsy maybe taken from the animals to determine their genetic status. Typically, the animals produced on both protocol 4 and 5 will be transferred to another PPL for experimental use or culled for tissue for laboratory research

Protocol 6 will allow administration of substances to modify gene expression by suppressing, enhancing, or switching on or off genes. These may be given by injection or orally. This includes administration of gene inducing or gene deleting substances to pregnant and lactating females as well as their offspring. Substances will be administered in diet or water, subcutaneously, or intraperitoneally. Implantation of a slow-release pellet subcutaneously may also be used. The purpose of this protocol is to produce animals for use on other project licences or tissue for laboratory research.

Protocols 1,2,3 and 7 are used to introduce animals into our specific pathogen free unit by embryo transfer or caesarean section, and to produce sperm/ embryos for cryopreservation (Protocol 1).

Protocol 1 is used to produced increased numbers of preimplantation embryos or eggs, mice will receive an intra-peritoneal injection of hormones, typically twice before being killed by a schedule 1 method and embryos/ ova harvested.
Animals on protocol 2 will produce surrogate mothers, after being rendered pseudo pregnant by mating with a sterile male, they will undergo surgical or non-surgical implantation of embryos whilst under general anaesthetic and receiving peri-operative analgesia.

Animals on protocol 3 will be vasectomised under general anaesthesia via cutting of the vas deferens by a scrotal approach.

Animals on protocol 7 will be mated and once pregnancy is confirmed receive a subcutaneous injection of progesterone up to three times, typically on pregnancy days 17-18 and then schedule 1 killed on day 19 and embryos harvested, and pups cross fostered to a recipient mother. This protocol will only be used for animals from trusted suppliers with a high health status to bring animals into our SPF unit.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the animals on this licence will be bred on the mild severity breeding and maintenance protocol (protocol 4) and will only ever suffer mild and transient pain or distress. A small number of lines will be bred on a moderate severity breeding and maintenance protocol (protocol 5). Some of these have the potential of developing tumours or neurological changes and weight loss. These animals will be monitored and at any signs of these adverse effects will be either transferred to another authorised project licence or culled for tissue.

Animals used on embryo recipient (protocol 2) and vasectomy (protocol 3) protocols will receive pain relief and are expected to make a full recovery within 24 hours of surgery. We do not anticipate adverse effects from the administration of transgene inducing or deleting agents (Protocol 6), but they could be affected by the genetic alteration. If any adverse effect is observed guidance from the NVS and the Home Office will be sought.

Animals on protocols 1 and 7 are expected to remain in the mild severity band, having received parenteral injections of reproductive hormones, and we do not anticipate any adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

85% of all mice allowed on this licence are expected to reach no more than a mild severity. 15% of the mice are expected to reach no more than a moderate severity.

What will happen to animals at the end of this project?

- Used in other projects
- Killed
- Rehomed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the research projects will initially involve the use of studies within the laboratory such as cell culture, human tissue assays, computer modelling to complement the animal work. Such details will be expected in the justification for the animals' use to be reviewed by the Animal Welfare and Ethical Review Body (AWERB).

However, studies within the laboratory cannot adequately model the complete array of molecular, cellular, physiological, and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes. To further understanding the pharmacological efficacy of human disease therapies and where studying such complex systems it will be necessary to follow events within a live animal model.

Which non-animal alternatives did you consider for use in this project?

Cell culture methods and computer models are used whenever possible; however it is not possible to replace all animal models and therefore this requires breeding of animals. Where replacement methods become available for projects supplied by this licence, this data is shared and disseminated by the Named Information Officer.

Why were they not suitable?

This project licence provides various GA animals that will be used under other project licences for research in live animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers have been estimated based on the usage over the previous 5 years on the licence this one will replace while taking into account the levels of work which are expected to be seen over the coming 5 years. The overall numbers have been reduced compared to the estimate given on the previous licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Mice will only be bred to order after discussing researchers' requirements for both mutant and appropriate control animals. The studies that the animals are transferred to are reviewed by AWERB to ensure that they are suitable, properly planned and are not using unnecessary numbers of animals. When breeding animals, online resources and guidance such as https://nc3rs.org.uk/breeding-and- colony-management will be used to ensure best practice is being followed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The strain and genetic background used for generating a new colony will be carefully considered to avoid producing unwanted mice. Animals will only be bred where a user requirement has been established, and the breeding programme will be subject to regular review to optimally meet demand.

Breeding will be optimised, wherever possible, to produce only the genotype required e.g. Homozygous breeding pairs. Spare animals will be made available for use on other scientific projects.

Only small numbers of animals within a breeding line will be kept. Expansion breeding will only take place to order and adequate time to produce the animals must be given.

Researchers will be encouraged to use male mice where possible.

Cryopreservation of sperm and embryos to archive lines will avoid wastage from the need to maintain colonies by continuous breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are widely used for work involving genetic alterations. The standard protocols, methods and reagents have been optimised for this species and there are acknowledged benefits from their use particularly as models for human disease as they share physiological pathways and almost all their genes are conserved with humans.

The methods chosen are all standard for this type of work.

Why can’t you use animals that are less sentient?

While other invertebrate models (Drosophila, C. elegans) maybe used to obtain information on basic physiological and pathological processes they lack the complexity seen in vertebrates. Mice offer tractable models with far closer anatomical, physiological, and genetic similarity to humans.

To maintain GA breeding lines, the animals used need to be of sexual maturity and therefore cannot be at a more immature life stage. A significant number of the animals bred undergo schedule 1 killing to produce tissue and should only suffer transient harm, and a proportion of these will be used for embryos production. Some of the animals will be used under terminal anaesthesia. Many animals will be used under other project licences that will be justified in their own right.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

GA strains will be managed, particularly those of a potential moderate severity to ensure they are not kept alive beyond their severity limits. Staff will be made aware by regular staff meetings, clear cage labelling, and training in how to detect any adverse effects.

Cage enrichment will always be provided, and cup handling has been introduced to reduce the stress caused to the animals during health checks, cage changing and procedures.

Monitoring post-operative and providing peri-operative care/pain management is required for all surgery animals. The anaesthetic used has also been refined from injectable to inhalation to reduce the amount of time the animals need to be under anaesthetic and to improve recovery times.

Needle use has been refined by introducing a policy of single use needles. Needles are now changed between mice during hormone injections.

Tissue obtained from identifying animals via ear punch is used to genotype most animals. Only where tissue cannot be used to genotype due to the requirement to know the expression level of the genes will blood sampling be used. Blood sampling has been refined from tail tipping to lancing to minimise the suffering caused to the animal. Due to these refinements in using the biproduct of identification methods and tail lancing, the requirement for tail tipping is now very rare.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the N3CR and Home Office guidance on best breeding practices at the following websites:

- https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/7735 53/GAA_Framework_Oct_18.pdf
- https://nc3rs.org.uk/breeding-and-colony-management

As well as keeping up with best practices on the following sites:

- https://wellcome.org/sites/default/files/wtd040017.pdf
- https://science.rspca.org.uk/documents/1494935/0/GA+passport+booklet.pdf/7050500f-4b6d-13ce- 9a6d-93fb01743ca9?t=1552661824197

Webinars and meetings will be attended on best breeding practices and colony management where relevant and available.

Best practice information is also disseminated by our Named Information Officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Published guidelines for best practice will be followed where possible, including:

- Home Office Efficient Breeding of GA animals assessment framework

https://www.nc3rs.org.uk/3rs-resources

- Norecopa EU ECVAM
- Animal Welfare and Ethical Review Body (AWERB) Home Office Animals in Science Regulation Unit RSPCA Animals in Science Department
- Understanding Animal Research
- Following and attending relevant seminars, meetings, and reading papers User groups

79. Targeting the tumour microenvironment of epithelial 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
- 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

Epithelial Cancer, Tumour microenvironment, Immunotherapy, Extracellular matrix, Macrophages

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop new ways of treating cancer by targeting the cells and molecules that control the tumour microenvironment. The term tumour microenvironment means all the cells that are found in a cancer, not just the malignant 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?

Cancers are not just collections of malignant cells but complex rogue tissues that contain many normal cells and molecules that are recruited and corrupted to help a cancer grow

and spread. This complexity, called the tumour microenvironment, can best be reproduced in animal systems such as mouse models of cancer. Over the past 10 years or so scientists and clinicians have learnt that treatments that alter the behaviour of these normal cells in the tumour microenvironment (e.g. immune cells, blood vessel cells) can shrink and, in some cases, eliminate cancers, even when they have spread around the body. These types of treatment are called biological therapies.

However the biological therapies are only effective in a minority of cancer types and patients. We need to understand how to improve on this. For instance, during the current project licence we have found that in patients and in mouse cancer models, standard chemotherapy drugs can stimulate immune cells to fight cancer but this is often not sufficient on its own; we need to find ways to enhance this.
Another example would be to 're-programme' some immune cells in the tumour microenvironment, that currently help malignant cells to grow and spread, into immune cells that destroy malignant cells.

Therefore, as part of our research, we need to use carefully selected mouse models that replicate as closely as possible their human cancer counterparts.

What outputs do you think you will see at the end of this project?

At the end of this project we aim to obtain new knowledge about the tumour microenvironment of cancer, especially some types of ovarian and breast cancer. We also aim to find new treatments, single, combination or sequential, that eliminate tumours or prevent relapse. This information will be translated to new clinical trials - for instance we are currently working on a 'window of opportunity' trial that will allow us to see if the anti-cancer effects we see in our new mouse ovarian cancer models are replicated in patient tumours. The patients will be given the experimental treatments for 2-3 weeks before surgery with a pre- and post-treatment (at surgery) sample that can be analysed and compared to the results in our mouse cancer experiments. The experimental treatments that we study are called 'biological therapies' and they include drugs that stimulate the body's immune system to attack the cancer and drugs that stop blood supply to cancers.

We will publish our work in international journals and present our work at national and international meetings (In the past 5 years there have been 12 publications that have resulted from work carried out completely, or partially in collaboration with others, under the current project licence).

Finally, we will make all the new information that we obtain freely available as well as any new mouse cell lines we develop. During the current project licence we have so many requests for the new mouse ovarian cancer cell lines we generated that we have handed them over to ximbio.com who are making them freely available to the scientific community.

Who or what will benefit from these outputs, and how?

Patients with cancer - we aim to have a least one clinical trial, based on our results, underway in advanced ovarian cancer by the end of the new Project.

Scientists and clinicians who study the tumour microenvironment from reading our publications and from listening to presentations at meetings.

Ovarian cancer researchers who are already obtaining our new malignant cell lines but will benefit from the new information about them that we obtain in the next five years.

How will you look to maximise the outputs of this work?

Collaboration is an important way to maximise our outputs. For instance we are currently working with collaborators on more efficient ways of imaging tumours in the mice. We plan to collaborate with experts on genetic changes in ovarian cancer to find new ways to combine the drugs they are developing with the biological therapies we are studying. We are also discussing a collaboration which will allow to understand how different clones of malignant cells change during growth and therapy.

We will publish unsuccessful as well as successful approaches and, importantly, we will make clear any differences between our mouse cancer models and the human tumour microenvironment that may influence our interpretation of the data.

Species and numbers of animals expected to be used

- Mice: 3000 female adult mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our work is entirely based on mouse models of cancer that most closely replicate the human tumour microenvironment. As the cancers we are modelling (e.g. breast and ovarian cancers) are generally found in females we will use adult female mice. We have developed several ovarian cancer cell lines that can be implanted into animals that are not genetically altered. We will also be using well- characterised breast cancer cell lines that grow in mice that are not genetically altered. Therefore approximately 80% of animals in this project will be normal healthy mice.

As we believe that the tumour microenvironment of different cancers share many similarities, the most promising treatments may be tested in models of other cancers, including some that affect men as well as women in which case we would use male mice.

Sometimes we may use immune deficient mice to understand the role of components of the immune system in the tumour microenvironment or response to biological therapies.

Typically, what will be done to an animal used in your project?

The most common experiment we will carry out in this protocol (>60% of studies) is the study of different drug combinations. In this type of experiment, tumour cells will be injected into the abdomen of adult female mice. Based on our experience with each tumour model, treatment will be started when tumour cells start to grow in the abdomen. Tumour growth will be typically detected by palpation (a method of feeling with the fingers for tumours during a physical examination of the mice), but an imaging session might be used at this stage.

In some of our experiments, we will use chemotherapy as a means of treating cancer. The minimum number of cycles of chemotherapy will be administered, typically three or six

cycles, depending on the model, by abdominal injection, once weekly. Other treatments will typically be administered sequentially for 6 weeks orally (generally once/day) or by abdominal injection (generally once/twice weekly). In slower growing tumour models where we see a therapeutic effect with certain biological therapies, providing there is no adverse effect, we may continue treatment for a maximum period of 16 weeks (i.e. up to 6 weeks of chemotherapy plus an additional 16 weeks of maintenance therapy totalling a maximum of 22 weeks). In some cases of experimental ovarian cancer, where we are interested in treating relapsed disease, we would stop treatment if all the cancer has been eliminated and recommence if the cancer returns.

Extended treatments will only be carried out where there is a clinical benefit to the mice. As we believe that the tumour microenvironment of different cancers share many similarities, the most promising combinations may be tested in several models and, in time, in models of other cancers, including some that affect men as well as women (in which case we would use male mice).

Some animals may be re-challenged with tumour cells injected in the abdomen. This experiment will be carried out rarely. Imaging will confirm that the mice are tumour-free before the tumour cells are re- injected. The aim of this type of experiment is to determine whether the mice have developed an anti- cancer immune response during the first course therapy, and that this twill prevent tumour growth when mice are re-challenged. Mice will not receive a therapy after the second challenge but will only be monitored for tumour growth according to our standard protocols.

To briefly outline a typical experiment in our breast cancer models, tumour cells will be injected under the skin of adult female mice. Mice are expected to develop solid mammary fat pad tumours, which can be measured using calipers. Caliper measurements and mouse weighing will be conducted three times a week to closely monitor tumour growth and allow us to collect and analyse the tumours when reaching a maximum volume of 1000 to 1250mm^3.

In addition to administration of cancer cells alone we also plan to give the mice combinations of cancer cells and normal cells called fibroblasts (the 'builder' cells of the body) as a way to control the way the tumour microenvironment develops. These fibroblasts may be genetically edited using molecular biology techniques in the laboratory prior to administration, in order to modify specific substances that we think may have an impact on anticancer immunity. Overall, by injecting different cancer and fibroblast cell combinations, we aim to build up a number of mouse breast tumour models, which reproduce distinct immune tumour microenvironment profiles.

Imaging may be incorporated to any study carried out in this protocol and will generally be used to confirm that most animals are developing detectable tumours before the start of a treatment. Imaging may also be used to follow up the response of the tumours to treatment. This would also reduce the number of mice we use, as it will allow us to study, in the same experiment, short term and long term effects of a therapy.

A blood sample may be collected to monitor the animal's health or the effect of the treatment. Generally, we would expect to run these checks up to once weekly over the course of an experiment. A volume of twenty microlitres (a small drop of blood - one microlitre is one millionth of a litre) is sufficient for this purpose. The blood sampling will not exceed what is indicated in the guidance from NC3Rs https://www.nc3rs.org.uk/blood-sampling-general-principles.

Mice bearing any of our cancer models will be culled at a pre-determined time-point whenever possible, or followed up until humane end-point is reached if the aim of the experiment is to find out if treatment prolongs life.

What are the expected impacts and/or adverse effects for the animals during your project?

From our past experience we predict that the planned studies will cause only mild or moderate discomfort. Animals are expected to return to normal activity within minutes after treatments such as cell injections or drug administration. We do not expect any adverse reaction related to drug treatments or their administration. Some distress is likely to occur in the advanced stage of tumour growth which could interfere with normal bodily functions. Indications of minor distress include weight loss up to 10%, reduced food and/or loss of appetite, hunched posture or hair standing on end. When animals have been subjected to minimally invasive surgery to administer cells in to the mammary fat pad/duct the recovery time is usually longer (2-3h after administration of anaesthesia and pain medication).

Animals in the protocol will be observed daily to assess for normal behaviours and general condition of the cages and their environment. If an animal appears to suffer, for example by demonstrating subdued behaviour when provoked, weight loss of 15%, small amounts of weight loss over a period of time without improvement, or continuous hunched posture, it will be humanely killed. An animal showing several repeated phases of intermittent hunched posture of a period of 48hr will also be humanely killed. At the end of each study when all results have been collected, all animals will be killed by humane procedures and their tissues used for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

A majority of the animals used in this project (90%) will be tumour-bearing and most will be subjected to single agent or combination therapies with the aim of eliminating their cancers or preventing return of the cancer. 25% of the animals will, in addition, undergo some form of imaging to assess their tumour burden. For these reasons, a majority of procedures will be of moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We study the tumour microenvironment which is a complex 'rogue' tissue that contains many other cell types, not just malignant cells. Cancers also evolve with time with the

different 'normal' cell types changing and being further 'corrupted' to help the malignant cells grow and spread. This is very difficult to model in a petri dish or tissue culture flask.

Which non-animal alternatives did you consider for use in this project?

Over the past 7 years we have been developing multi-cellular human models of the tumour microenvironment that grow in the lab and we have just published the first two papers on these models. Alongside the malignant cells we can add some types of immune cell, fibroblasts and cells that usually line surfaces in the body, to the models. These models now have five cell types in them and are proving to be complementary to our mouse models. However, we do not think we can ever totally replicate the complexity of the human or mouse tumour microenvironment and we do not think the multi-cellular models will ever replace mouse models. However, as will be described below, the multi- cellular models are part of a platform of cancer models that will allow us to reduce the use of mouse cancer models during this project licence period.

Why were they not suitable?

The multi-cellular models cannot replicate the cellular complexity of the mouse cancer models, nor can they be grown over the longer periods of time needed for some of our experimental treatment schedules.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimates are based on our knowledge of the natural history of the mouse models we use and experiments (published and unpublished) that have given us statistically significant results. We have extensive experience of murine cancer models and over the past 15 years have collected data on the most suitable models and number of animals per group to generate reliable data. The studies in this project will utilise these models while advancing our work to improve on both models and therapies. The work is now at a stage where we need to focus on the interactions of our novel therapies with the tumour microenvironment and the host immune system. We estimate up to 3000 animals will be sufficient for testing efficacy and bio-distribution of various combination therapies (including novel therapies and conventional therapies). However, these are the maximum estimated number of animals for the duration of the project licence and in each study an exact calculation will determine the numbers to achieve statistical significance.

We will also refer to the NC3Rs Experimental Design Assistant: https://www.nc3rs.org.uk/experimental-design-assistant-eda

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are reducing the number of mice used in our experiments because we have now developed a 'platform' of cancer models, where the mouse cancer models are only one resource. For instance we now have extensive human *'in silico'* (computer) databases from our own research projects and publically available sources. We have similar data for some of our mouse models. These sets of information allow us to test hypotheses using computer-generated programmes and guide our mouse and other types of experiment. Then we have the multi-cellular models described above, and finally we have developed a 'tissue slice' technique where we can, over short periods of time, treat human tumours with an intact tumour microenvironment. We also use tissues in which we have removed all the cells to help study immune cell interactions with the cancer extra-cellular matrix (the stiff protein network that binds our tissues together).

The impact of of all these models can already be seen in the reduced number of mice we will use in the next Project period, and the fact that because of our new models we have eliminated the breeding aspects of our experimental designs. For instance there is 60% reduction in the estimate of the number of mice we will use in Protocol 1 and we will not use any mice for breeding purposes.

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 very important in terms of reduction, especially when we are assessing combination therapies. As we now have extensive data on the molecules, cells and extracellular matrix of the cancers we study, we can first conduct computer-based studies and work in the other human tumour microenvironment models in the lab before moving to the mouse experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are studying the tumour microenvironment therefore we need mouse models that develop cancers in the correct microenvironment. These are called orthotopic tumours. For instance, for ovarian cancer models we inject the malignant cell lines into the peritoneum and for breast cancer models we inject into the mammary fat pad/duct. We continue to seek improvements to our techniques, this includes using ultrasound echo-guided injections to deliver treatments and cells directly in to target organs or tissues reducing the need for surgical manipulation that can result in pain or distress to the animal, which may affect the immune system and potentially confound experimental results.

This project will mostly use not genetically altered animals, however we do not exclude the potential use of immuno-deficient mice to compare tumour development and profiles in the presence or absence of a functional immune system.

Why can't you use animals that are less sentient?

We cannot use animals that have been terminally anaesthetised because some of our models evolve over several weeks and months. We try to replicate as closely as possible human cancers that generally grow slowly. Also tumour microenvironments do not develop in days; it takes time to recruit the other cells and for important components such as extra-cellular matrix and blood vessels to develop in cancers.

We can only use mice for these models as the malignant cells will only form tumours in mice and it is not relevant to do these experiments on immature life stages of mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The best way we feel to minimise harm is to refine our non-invasive imaging methods. This means that we will be able to find out at a very early stage when a tumour is growing too fast or has spread somewhere that will cause increased suffering. We are collaborating with a number of scientists to refine our experiments in this way.

Increased monitoring is also a vital component of our procedures to minimise suffering. We have and will continue to refine our palpation scoring method for the growth of internal tumours in our ovarian cancer models. We have also developed a single sheet that provides a clear overview of the mouse's welfare record including a history of body weight, palpation scores from first detection to the most current as well as a place to indicate if there are any other noticeable health issue to monitor. Having all of this information available at any time to all technicians and researchers involved with the study allows for a more accurate, informed decision to be made more efficiently. Health issues requiring action are reported to relevant Named Persons and the responsible personal and project licence holders using the BSU database management system. We are exploring this further to see if/how we can effectively incorporate detailed monitoring records including observations on to the database as this would help greatly, enforcing efficient communication and notification of health issues between all those involved in a study.

When interventions are repeated, we are looking in to the potential for acclimatisation, which may reduce severity including positive reinforcement training, or 'rewards' following procedures.

We also strongly believe in the benefit of a consistent approach to monitoring. Elements contributing to consistency include:

Incorporation of multiple expertise, experience and priorities – a 'team approach'

Training in using the day-to-day assessment protocol (including the common terminology used to describe observations)

Expertise on animal health, welfare and behaviour

Regular review of outcomes

Communication between all those responsible for conducting the study and monitoring the animals (top-down, bottom-up, between and within)

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642

https://iqconsortium.org/images/LG-3Rs/IQ-CRO_Recommended_Dose_Volumes_for_Common_Laboratory_Animals_June_2016_%282%29.pdf

Refining procedures for the administration of substances, Report of the BVAAWF/ FRAME/RSPCA/UFAW Joint Working Group on Refinement, D.B.Morton et al; 2001.

A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes
and Volumes, Diehl et al, J.Appl. Toxicol. (2001) 21, 15–23.

The directions for reporting of actual severity levels, for example; Smith et al., Laboratory Animals 2018.

Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0 Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020) Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLOS Biology 18(7): e3000411. https://doi.org/10.1371/journal.pbio.3000411

M.F. Festing, Design and statistical methods in studies using animal models of development, ILAR J, 47 (2006) 5-14. 18

J. Charan, N.D. Kantharia, How to calculate sample size in animal studies?, J Pharmacol Pharmacother, 4 (2013) 303-306

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Areport by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.) http://www.lasa.co.uk/publications/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have an effective 3Rs network through the NC3Rs and attend our establishment 3Rs events as well as those within our network. Staff attending conferences (such as the IAT Annual conference) will bring back best practice in terms of the 3Rs and new techniques and we will keep abreast of the literature to ensure that we use the best and most reproducible methods.

An example of an advance highlighted to us by NC3Rs and our resident Named Veterinary Surgeon which we have implemented is tunnel handling. Tunnel handling has had a noticeably positive effect on reducing animal stress and anxiety prior to carrying out procedures.

80. Understanding host-pathogen interactions in zebrafish 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

Infection, Immune cells, Zebrafish, Antimicrobial resistance

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, adult, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how our immune defence system fights infections and what goes wrong when it can’t control infections leading to disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infectious disease is the world’s biggest health problem. Bacterial and fungal infections are beset by the growing problem of antimicrobial resistance where the germs are becoming resistant to existing treatments (eg, antibiotics). This project will advance our knowledge of the immune system and how infections happen in people whose immune systems to not work correctly. Using this knowledge, we will try and find new ways of treating infections by helping the immune system. Boosting the immune system is a treatment strategy that could work alongside or replace existing antibiotics, reducing the need for new antibiotics.

What outputs do you think you will see at the end of this project?

Infectious disease is still the world's biggest health problem, and the speed of emergence of antibiotic resistance has vastly outstripped our ability to identify new antibiotics. The consequences of this reach well beyond the confines of infectious disease – without antibiotic therapy, we could not safely perform anything other than the most simple of surgery, nor give anti-cancer chemotherapy. A return to pre- antibiotic healthcare is a real threat and amongst the gravest threats to human health.

In vivo visualisation and manipulation of host-pathogen interaction will allow considerable advances in our understanding of how pathogens attempt to evade their killing by host phagocytes, how the host tries to prevent that evasion. This area has considerable potential for identifying completely new ways of treating infectious disease, and critical in an age of widespread antimicrobial resistance. For example, blocking pathways that bacteria and fungi use to prevent host killing could lead to new treatments for infection, ones that might avoid the pitfalls of resistance associated with traditional antibiotics.

As with our previous work, we will publish our findings in as journal articles in an open access manner with maximum impact and will present our work at national and international scientific meetings. We will also disseminate our findings to the public to improve the public awareness of science.

Who or what will benefit from these outputs, and how?

Groups that will benefit from this research programme include:

Scientists directly involved with the project

The short-term benefits will be to our research group and to other researchers in our fields and in closely allied areas e.g. immunology and microbiology. Immediate beneficiaries of this project will be the scientists working on the project (PI, RA and PhD students). With the opportunity to apply new approaches and techniques with Zebrafish models there will be career development opportunities. Researchers will be trained in the priority skills areas of whole organism physiology, quantitative skills (proteomic analysis), and interdisciplinary (particularly imaging).

The scientific community

Academic researchers in several research areas will benefit from this work including those working on pathogenesis of infections, inflammation and researchers in immunity diseases field, zebrafish research community and researchers in cell signalling and molecular biology fields.

Patients and wider public

In the medium-term our programme of work may identify potential targets and leads for further development that, in the long-term, may result in new ways of treating infections disease. Patients and the wider population will benefit from this research if we can identify novel pathways for future research and ultimately, targets for therapies to lessen the burden of infectious diseases.

Industry

There may also be short term benefits to industry through the availability of new knowledge in how pathogens evade killing. This work will identify drug targets that could be used to treat a huge unmet clinical need. It is likely that this will be of interest to pharmaceutical companies, large and small. We will develop strong collaborative links to enable commercialisation of any targets as they arise.

How will you look to maximise the outputs of this work?

We will maximise our outputs by:

effective collaboration with experts in the field of zebrafish inflammation and immunity, which will extend our skill set and facilitate access to advanced technologies and ensure that we are using the most cutting-edge transgenic/mutant tools to answer our scientific aims

dissemination of new knowledge at key meetings and conferences

dissemination of our work more widely through press release via university media team

publication and sharing of both successful and unsuccessful approaches where appropriate (eg via F1000, BioRxiv, Wellcome Open Research, or NC3Rs Gateway platforms)

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 21500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 have many properties which make them the only available system to answer the scientific aims of this PPL. Zebrafish are vertebrates that have a comparable genome to humans. Zebrafish larvae are almost completely transparent, enabling unrivalled visualisation of in vivo host-pathogen interaction. Major cellular components of the innate immune system are present in the larvae and share direct homology to mammals. Zebrafish are readily amenable to genetic modification and there are a host of emerging genome editing technologies available (eg CRISPR-Cas9). Larvae are small, allowing pharmaceutical approaches to be performed by immersion in 6-96 well plates format, (akin to a cell model system) while maintaining the benefits of whole-organism investigation.

The zebrafish as a model of infection is emerging as a favourable alternative to traditional mice models. We have previously used the zebrafish model successfully, and have demonstrated modulation of inflammation and infection using many of the assays required for this PPL. Fish are ranked as the lowest order vertebrate used in animal research (in terms of pain and suffering), therefore the neurology and suffering may be considered less than higher vertebrate systems. Under 5.2 days post fertilisation zebrafish embryos could replace current rodent models of infection, thereby contributing to 3Rs.

Zebrafish infection models represent exciting and viable options for investigating the pathogenesis of infection in a whole organism setting.

Typically, what will be done to an animal used in your project?

Zebrafish will be used to generate mutant and transgenic lines of proteins involved in the immune defence against infections. The roles of these proteins during infections will be tested by deletion in zebrafish one at a time using gene-editing technology. Experiments will almost exclusively be performed on animals before they become protected (<5.2 days post fertilisation at 28 degrees Celsius). These procedures involve microinjection techniques to deliver RNA and protein material to induce mutation at 1 cell stage zebrafish embryos. We will then characterise the effect of these mutations in zebrafish on the response to inflammatory response induced by infections. This involves advanced imaging as well as gene expression and biochemical analysis.

The vast majority of zebrafish used (over 20,000) will be used for breeding (up to mild severity) to produce zebrafish larvae for maintenance of transgenic/mutant lines, or to use in experiments before they become protected. Adult animals will be kept for between 2 and 3 years (their healthy lifespan) and culled by overdose of anaesthetic before they show signs of significant aging. Our experiments are designed to understand how infections are cleared from the animal and most animals will therefore experience only mild severity.

What are the expected impacts and/or adverse effects for the animals during your project?

Infections cause adverse effects on larval fish, including excessive inflammatory phenotypes that lead to oedema. Greater than 95% of larval infections will be halted before the onset of protection at 5.2 days post fertilisation.

The generation of mutant lines in the immune system could lead to immunocompromise and susceptibility to infections. To minimise the risk of this, mutants in genes likely to lead to this will be kept as heterozygotes (rather than homozygotes), and closely monitored for infections. These mutants will not be kept to old age, with new generations generated after 2 years to ensure humane endpoints before age susceptibility to infections (that can happen if kept beyond 3 years of age).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: 95%

Moderate: <5%

What will happen to animals at the end of this project?

- Killed
- 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?

Infection occurs in a complex tissue microenvironment milieu that cannot currently be replicated in in vitro systems. Investigation of the innate immune response to infection requires four main cell types (neutrophils, macrophages, bacteria, and endothelial cells) along with the surrounding tissue microenvironment. Emerging evidence also implicates the neural system in infection, further highlighting the need for a holistic approach. To study interactions of these cell types in a non-living environment would not provide the comprehensive environment in vivo research allows.

Although this project involves animal work, the proposed research will have 3Rs impact in the following ways. My proposal uses the well-validated, widely-published Mycobacterium marinum zebrafish model to investigate novel areas of TB research. It has the potential to replace rodent and other mammalian models (eg rabbits) to investigate TB pathogenesis. In order to avoid mammalian models, I will test findings from zebrafish in innovative human cell models. A benefit of the zebrafish Mm model is that Mm is a natural fish pathogen, unlike in murine models of TB, therefore the investigated host response to infection is a naturally occurring one. Further expansion of this exciting model will compliment other excellent TB research currently undertaken in the UK. Publications and dissemination at conferences will increase and enhance the profile of the zebrafish to wider infection and immunity fields. It will highlight the conservation of pathways in mammals and fish and promote the zebrafish as a genuine alternative to mammalian research.

Which non-animal alternatives did you consider for use in this project?

Infection and inflammation occur in a complex tissue microenvironment milieu that cannot currently be replicated in in vitro systems.

In vitro cell systems to investigate neutrophil and macrophage migration towards infectious/inflammatory stimuli are currently available, but are only useful for asking very specific questions about chemoattractants. Where possible, we will use these models using our findings from zebrafish models and to inform our zebrafish investigations, reducing the animal numbers required. For example, we are performing in vitro work using microfluidic chambers on human blood cells migration towards individual chemokines. However, these cannot completely replace in vivo systems as they lack the complex microenvironment that occurs during infections.

We are also performing in silico computer simulations of processes involved in phagocytosis (intake) of pathogens. However, these are only appropriate to answer specific physical properties of the immune cell (eg, how much actin cytoskeleton is required) currently, but further development of these models will help limit animal use and may eventually be a future replacement.

Why were they not suitable?

The approaches above significantly help to provide more concise information on the

genes/proteins needing to be targeted in vivo but they cannot mimic the actual cellular response occurs in the animal to this challenge.

The innate immune response to infection and this requires four main celltypes (neutrophils, macrophages, bacteria, and endothelial cells) along with the surrounding tissue microenvironment. Emerging evidence also implicates the neural system in infection, further highlighting the need for a holistic approach. To study the interactions of these celltypes in a non-living environment would not provide the comprehensive environment in vivo research allows, which is an absolute requirement to understand the cellular mechanisms involved in the immune response to infections.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experiments that require animals we will use previously collected datasets to predict the minimum group sizes required for statistically relevant results. For any new experimental models we will perform pilot experiments with small numbers of animals to interrogate the biology to inform future work. We will consult with other biologists and statisticians when planning these experiments. Numbers of adult zebrafish required are calculated based on a requirement to have enough healthy adult fish at any one time to generate the larvae required for experimentation. Although large numbers of larvae can be obtained on one spawning, fish cannot then spawn for 2 weeks, meaning a rotating stock of fish is needed to ensure embryo supply. To minimise animal use, once experiments have been performed sperm will be frozen (for future IVF) and stocks will not be maintained.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will aim to use the minimum number of both protected and non-protected animals. Almost all animals will be used for breeding purposes within their healthy lifespan and when these animals are not required we will minimise the number of fish required to maintain the line. For protected/non- protected animals we have used published datasets to predict groups sizes needed to generate data which will produce statistically relevant outcomes. Where appropriate we will use the NC3R’s Experimental Design Assistant to consult on sample sizes for individual experiments to ensure only the minimum number of animals required is used. We have performed pilot experiments in order to ascertain key timepoints. We have searched on-line databases to identify bioinformatic data which will allow us to narrow the range of hypotheses and sometimes to refute them without experimental work.

Most of proposed fish experiments are performed on larvae before the onset of independent feeding– these larvae are not considered protected by the Animals (Scientific Procedures) Act. Procedures for making mutants in zebrafish are mild severity. Genotyping of Adult fish is done on anaesthetised fish (small tail biopsy which regrows).

To minimise animal use, once experiments have been performed, sperm will be frozen (for future IVF) and stocks will not be maintained. Spare larvae are used for other experiments or shared with other members of the laboratory for reasons of efficiency.

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 fecund, so we are able to obtain large numbers of embryos <5.2dpf from a small number of parents. To minimise animal use, once experiments have been performed, sperm will be frozen (for future IVF) and stocks will not be maintained. Pilot studies are performed initially to inform on the optimum number of animals needed per experiment to see a significant effect. Spare larvae will be used for other experiments or shared with other members of the laboratory for efficiency.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish have a comparable immune system to our own, with similar types of white blood cells, meaning findings are relevant to human disease. However zebrafish have a much lower neurological capacity than other animals used in immune studies and are therefore the most refined model for this project.

Fin clipping and gamete expression methods rarely involve discomfort, but for all manipulative procedures animals are suitably anaesthetised (primarily for restraint). Anaesthetic is delivered by immersion (so no instrumentation is required) using an anaesthetic protocol suitable for the procedure and following best practice.

The methods for generating mutants and transgenics are optimised to minimise numbers and adverse effects are minimal. Generation of mutant/transgenic animals with tumour propensity will be based on well established techniques for mutation and transgenesis, and the animals will not suffer directly due to the techniques. However, the infections might lead to suffering and needs to be closely followed.

Where feasible, early genotyping using either embryonic fin clipping or a zebrafish embryonic genotyper (ZEG) system will reduce the number of larvae raised as we will only raise those of the required genotypes.

In any infections of protected animals we will minimise harm by culling animals that show excess clinical signs of infection. Over the course of the current licence we have refined our experimental readouts to mainly use <5.2 days post fertilisation for infection work, to minimise pain, suffering and distress. We will closely monitor all animals and will treat or cull by overdose of anaesthetic if needed.

Why can't you use animals that are less sentient?

We have carefully chosen the zebrafish as the most refined animal model for these studies. Fish are ranked as the lowest order vertebrate used in animal research (in terms of pain and suffering), therefore the neurology and suffering may be considered less than higher vertebrate systems, especially at the under 5.2dpf stages that are investigated here. We will only go over 5.2dpf if the biology requires it (eg, if T cell responses are required for the biological outcome being investigated. T cells only develop beyond 5.2dpf). The translucency of zebrafish larvae and transgenic fluorescent lines allow non- invasive imaging allowing collection of dynamic data over time without surgical intervention.

Drosophila (fruit flies), do not have both macrophages and granulocytes (neutrophils) therefore cannot model the human innate immune response to infection fully.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise suffering and discomfort the animals will be monitored and when there is any concern advice will be sought from the named veterinary surgeon and/or the Named Animal Care Welfare Officer and appropriate action taken. I also have access to a NC3Rs regional programme manager with whom I will consult as well as attend relevant workshops/seminars to learn about the latest developments in the 3Rs. This keeps me updated with evolving 3Rs best practice. For any experiments performed beyond the onset of independent feeding, larvae will be monitored regularly and individual experiments will be evaluated before further larvae are used in this protocol. The advantages and risks of this approach will be evaluated after each larva is used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To the best of my knowledge there are no published best practice guidelines for the zebrafish work in this project licence. However we use philosophies of experimental design advocated by the likes of Festing and Wurbel in order to refine our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The research groups will stay informed about advances in the 3Rs through communication channels including zebrafish user meetings, NC3R symposia, updates from NACWOs that attend animal husbandry/welfare conferences, collaborations, and through direct advice and consultation with the NTCO, NVS and NACWOs.

I am a current member of an NC3R funding panel and am therefore a representative of NC3Rs and must stay up to date on current advances of the 3Rs in that role.

81. Discovery and development of treatments for immunological 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

Autoimmune disease, Rheumatology, Dermatology, Immunological disease, Gastroenterology

Animal types	Life stages
Mice	adult, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This licence has two aims:

To develop and validate animal models that can further our understanding of basic mechanisms involved in immunological disease and enable us to test new therapeutic agents.

To use the models developed above to identify novel treatments that will impact human immune 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?

Despite decades of research, millions of people live with serious immune mediated disease such as colitis, arthritis, systemic lupus erythematosus (SLE) and atopic dermatitis. For example rheumatoid arthritis, which is an autoimmune disease resulting in painful swollen joints and loss of movement, affected nearly 20 million people worldwide in 2017 (Safiri 2019) with more than 1 million new cases being recorded each year. Other autoimmune diseases such as lupus (SLE) can have devastating effects on patients lives, including debilitating symptoms which impact their ability to work. Allergic inflammation can also lead to the development of serious immune mediated disease such as atopic dermatitis, where the inflammation and pruritus (itch) present significant unmet medical need. Current treatment options are not effective in treating all patients, and many have significant dose limiting side effects (e.g. steroids). We believe we can identify novel treatments to address the symptoms and underlying mechanisms which cause these conditions, and addressing this unmet clinical need is a high priority for the medical research community.

Safiri S, et al. Ann Rheum Dis 2019;78:1463–1471

What outputs do you think you will see at the end of this project?

This Licence will enable us to investigate potential new mechanisms involved in multiple immune mediated diseases and test our novel treatments in simple and complex models, with the ultimate output being a new medicine progressing through to human clinical trials. Simple mechanistic models will be used to demonstrate the role of a particular pathway or system, for example using a treatment known to activate a specific pathway, and then using a novel treatment to block the effect. The more complex models, which engage multiple interacting systems but do not necessarily replicate a human disease, will be used to provide additional information about the likely effect of our treatment on a disease. For example, we may gain more information by seeing how a new treatment blocks the development of skin inflammation in an immune driven model, having previously tested it for activity using a selective stimulus. The outputs of these studies will contribute to active decisions to progress projects and novel treatments along the development path towards clinical testing, or to cease work on pathways or therapies which appear to lack benefit. These studies occur very early in the research pipeline and it can be difficult to publish due to concerns about patent life and intellectual property, but we would expect to share our findings as much as we can with the scientific community.

Who or what will benefit from these outputs, and how?

As an organisation our focus is on delivering life changing medicines to patients, and this licence will contribute to that overall goal by enabling us to test new therapeutic hypothesis in vivo relevant to new treatments for immune mediate diseases. Along the journey to delivering that clinical goal, we will also generate new scientific findings, and build our knowledge of the pathways and biological systems involved.

Drug discovery and development can take years if not decades, so novel treatments tested under this licence are likely to enter human clinical trials after the 5-year lifetime of the licence. However, treatments tested under previous licences with the same goal are now in clinical phase testing and we are confident that this will continue.

How will you look to maximise the outputs of this work?

We have an open culture and interactions between our scientists and those from other research organisations are encouraged. Whenever possible we regularly share our

findings at academic conferences and symposia, but due to the commercially sensitive nature of the work we are unable to be open about our very early and novel findings until appropriate patent protection is obtained. We do share openly information regarding our animal study techniques and practices with our peers to maximise animal welfare, and are committed to supporting the 3Rs.

Species and numbers of animals expected to be used

- Mice: 8000
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The overwhelming majority of these studies will be performed in adult mice, as this is the species most commonly used in the disease model studies we will carry out. Mice are used in those models as they are a mammalian species with a similar (but not identical) organ anatomy and immune system as compared to humans. Although immunological diseases can affect patients at any age, the majority of rodent studies are performed in adult individuals (ie post-pubertal) as rodent development is very rapid and animals become sexually mature in a matter of weeks. Rats will be used rarely, when the disease model is performed in the rat, or there is some other technical need (such as the target system in the rat being closer to the human in its properties).

Although it will be very rare, we may occasionally need to understand the responses of the immune systems in aged animals, which for mice and rats equates to 1 year or older. This will only be done where there is a clinically relevant question or scenario that requires it.

Typically, what will be done to an animal used in your project?

In a typical simple immune challenge experiment, adult mice would be implanted with identification microchips under anaesthetic and a few days later weighed and dosed (the dose given being related to the weight of the animals). The dose may be an oral liquid, delivered using a round ended dosing tube attached to a syringe which is placed into the throat of the animal to ensure all the material reaches the stomach. Alternatively, the dose may be given as an injection using a hypodermic needle under the skin, or into a vein or into the abdominal cavity. Animals are returned to their cages and then at a set time after dosing, they may have an immune stimulating substance injected into the skin under general anaesthesia. Once the animal has recovered they would be returned to their home cage. At some time later the animals may be taken out and restrained in a small tube for a few minutes while a blood sample is taken from the tail using a small hypodermic needle. Usually no more than 3 samples are taken from the tail vein in the lifetime of the animal, and only if the samples needed are very small.
Blood samples may be analysed for signals such as immune cell activation, or for drug content. The final blood sample is usually taken under deep non-recovery anaesthesia, when a hypodermic needle is placed directly into the heart to take a large volume of blood, followed immediately by killing the animal. At this time, after confirmation of death, the skin would be dissected to allow analysis of the injected region.

Variations on this design may include the injection or topical application of substances which can cause an itch response, and we will monitor the animals to observe the scratching frequency and other parameters in order to determine if our treatment has modulated the response. Alternatives to injection into the skin may be topical application to provoke an immune response and local inflammation.

Some studies may involve another body site such as a subcutaneous air-pouch, which is generated by injecting sterile air under the skin on the back of the animal. Once the pouch is formed, inflammatory cells can be recruited and activated locally in the pouch following injection of an immune stimulating substance.

Stimulation of a pathway present in circulating cells or a specific organ can be achieved by an injection into a vein, or abdominal cavity. Activation and inhibition can then be monitored by peripheral sampling of blood or terminal sampling of body fluid and tissues.

Treatments in the studies above will typically be given by injection or through dosing into the stomach, but in a minority of cases we may also use a surgically implanted drug delivery device (pump) to do this. In some studies we may inject cells from another animal or which have been grown in a test tube, to enable us to track the movement of cells during immune responses, or to have a role in the immune response itself (e.g. using cells which respond to a known allergen).

What are the expected impacts and/or adverse effects for the animals during your project?

Animal dosing may cause some brief pain, such as when a hypodermic needle is used to dose under the skin or into a vein, the abdominal cavity or air-pouch. This is brief in nature and animals are not expected to show any signs of ongoing discomfort afterwards. Similarly, the needle used to take a blood sample is likely to result in fleeting pain or discomfort but should not have a lasting effect.
Injection into the skin (intradermal) is performed using general anaesthesia to reduce discomfort and allow greater control of the injection.

The sensitisation and challenge models we propose are expected to produce local inflammatory responses, which are likely to include swelling and increased sensitivity (hyperalgesia), and in the case of pruritic responses, irritation that evokes scratching (itch). Overt pain is not an intended consequence of these models and animals will be monitored to limit the severity experienced. Animals may exhibit clinical signs associated with inflammation and illness such as reduced grooming (and/or over grooming of inflamed or challenged areas), reduced social interaction and lowered body temperature. These effects may last over several days, for example following sensitisation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In this Licence the severity experienced by an individual animal is a function of the immune modulating steps and the drug delivery steps. The majority (>60%) of both mice and rats are expected to experience a severity of Moderate due to the inflammatory and irritant challenges. We have run most of the proposed studies under a previous project licence

(e.g. HDM induced ear inflammation, agonist evoked itch, air-pouch) and this estimate is based on the potential for therapeutic effect to ameliorate the inflammation, control injections/challenges which would be expected to have minimum impact on animal welfare and the use of drug-dosed, non-challenged controls which are required in many studies. These factors will bring many animals down into the Mild severity band.

A minority of animals (estimated <10%) may experience Moderate severity due to recovery surgery for implantation of drug delivery pumps under the skin.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not yet technically possible to eliminate all animal studies from our preclinical research programmes as the complex interplay of physiological systems including the innate and adaptive immune systems and the nervous system cannot be replicated using in vitro technologies. One reason for this is that we are working with highly novel mechanisms and need to ensure that in vitro findings can be extrapolated to our in vivo settings.

Which non-animal alternatives did you consider for use in this project?

There are multiple stages of non-animal screening of new treatment strategies as well as novel molecules themselves that are in place before testing in rodents takes place. Depending on the type of new therapeutic agents we will have performed tests (both using computer modelling and lab testing) to understand the physical properties which can affect drug absorption and how rapidly it could be broken down in the body. Cell based systems are used to assess how for example liver cells might process and break down a molecule. The biological pathways we are studying are investigated using multiple human and animal cell based systems to ensure that the molecules we are going to test will modulate the target pathway or system.

Why were they not suitable?

The information gained from these non-animal tests cannot account for the complex interactions that take place inside a whole animal with multiple organs, cells and enzymes which could affect the way a drug molecule moves around the body, is processed or broken down and is eliminated from the body. In addition, the biological mechanisms and pathways we are seeking to modulate are both complex, involving innate and adaptive immune responses and interactions with whole organs and body systems, and highly novel and we want to ensure that the behaviour of these is physiologically relevant and not an artefact arising from cell or tissue culture conditions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These estimates arrived at based on current expectations (how many animals per study, how many studies of each type per year) and allowing for anticipated changes in demand. For each protocol 1-4, the estimate is 2000 mice over 5 years, which is 400 mice per year per protocol.

For rats, the 1500 figure is composed of 250 rats over 5 years (ie 50 rats per year) for Protocol 1 and 2, and 500 rats over 5 years for Protocols 4 and 5 (ie 100 rats per year per protocol)

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Before performing in vivo studies under this Licence, we will have conducted extensive in vitro research studies to confirm as far as possible that the agents we will use are pharmacologically active against the species specific target and have sufficient potency or affinity to have a biological effect in vivo. This reduces the risk of performing studies with agents that cannot be progressed. Prior to the studies described here, experiments are carried out to ensure that the dosing will be effective, by measuring the amount of treatment in blood after administration. This de-risks again the potential for using new therapeutic agents which are unlikely to achieve active concentrations at the target tissue or organ. Careful consideration is given to the number and size of all groups, and the overall goal is always to use the minimum number consistent with the scientific objectives. As we are testing novel materials it is necessary to include control groups to ensure the interpretation of the data is clear, which reduces the need for follow-up studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The animals we use for these studies are usually standard strains obtained from commercial suppliers which maximises the efficiency of production and animal usage. Occasionally we will use genetically altered animals bred for scientific use and the numbers are carefully monitored to ensure overproduction and wastage is minimised. As described elsewhere in this application, pilot studies will be performed to inform our use of power calculations as well to determine the optimum effect window and kinetics 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models described here are mechanistic and are designed to allow us to study particular pathways or systems which are involved in a disease. For example, by using a simple model of cell recruitment into an air-pouch, we can study the movement of inflammatory cells (such as T-cells, monocytes and neutrophils) into the pouch and the mediators and enzymes they release, and look at the effect of blocking those mechanisms. Using this approach enables us to study pathways and pharmacological effects without having to use animal models of arthritis in the first instance. The animal welfare burden in arthritis is greater as the animals can experience pain and discomfort from swollen and inflamed paws, and this licence is not requesting permission for that model. The pouch cannot replace all arthritis studies but does allow us to study the biology and pharmacology of these mechanisms in a lower severity model, reducing the risk of proceeding into a arthritis study with an ineffective new treatment. In a similar way, we can use short/simple (hours-days) mechanistic studies of skin inflammation and itch prior to complex models of immune mediated skin inflammation (days-weeks) to focus our studies. This approach minimises the use of models with a higher welfare impact.

Why can’t you use animals that are less sentient?

These studies are used to provide critical information to project teams regarding the efficacy of our new treatments, and will inform decisions regarding the progress of these treatments into more complex models of disease (which may not fall under the remit of this licence) and eventually into human clinical studies. Using species that are less sentient introduces a significant risk that the information we obtain is not relevant and could lead to wasted animals in the disease models. Terminal only studies would be of limited duration and risk giving information that is compromised as for example blood flow to the skin, liver and other organs may be different under anaesthesia and could affect the measurements, and the presence of anaesthetic could interfere with the mechanisms (or limit our ability to make measurements such as scratch behaviour).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals are kept in modern well equipped facilities staffed by experienced and motivated scientific and welfare personnel. Animals are checked at least once daily when not on a study and at least twice daily once dosing and sampling have started. When surgery (under general anaesthetic) is used for implantation of drug delivery devices, peri-operative pain relief (analgesia) will be given as standard.
Hypodermic needles are always discarded after a single injection so that blunted needles (which can cause unnecessary tissue injury and pain) are not used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA Good practice guidelines; AAALAC programme

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a Project Licence holder I am engaged with local and national 3Rs groups and events and am kept informed by my NIO of relevant new information.

82. Integration of DNA replication stress response and genome stability maintenance pathways

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

DNA replication, cell division, Cancer, Genome stability

Animal types	Life stages
Xenopus laevis	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this work is to gain greater understanding into the mechanisms that cells use to respond to DNA replication stress and how these mechanisms work to ensure that DNA synthesis and cell division are regulated to prevent the generation of genome instability which ultimately can lead to carcinogenesis in humans.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important as it will provide further insight into the ways DNA replication stress develops in cells and how this process drives the transformation of a normal cell to a tumour cell in addition to other associated changes in cell morphology. It has now been established that the genome instability observed in most human cancers is driven by the DNA replication stress that occurs as the transformation from normal cell to a cancer cell progresses.

The work supported by this project is likely to identify additional components and pathways involved in to the cellular response to DNA replication stress. In addition to contributing to extending our knowledge of how DNA replication is regulated and although not directly attributable to this project, it is likely that such insights will also prove vital for identifying the key protein factors important for these pathways and in doing so identify new targets that will enable the development of new drugs or therapies to treat a range of human diseases in addition to human cancer.

What outputs do you think you will see at the end of this project?

Outputs from this project are anticipated to be disseminated through the publication of research articles in general interest and specialised scientific journals. In addition to these the results and findings will also be disseminated to the research field via seminars and presentations at scientific meetings. These presentations will mainly be to other scientific researchers with interests in DNA repair, cancer biology and DNA replication at national and international scientific meetings but there is an anticipation that results will also be presented to a lay audience at meetings organised by the various funding charities to provide the volunteers who raise money and donate funds to the charity with details of the work they are supporting.

Who or what will benefit from these outputs, and how?

The work supported by this project is basic biological research so the immediate benefit will be the advancement of scientific knowledge regarding the ways that DNA Replication stress and DNA Damage Response pathways are regulated and how defects in these pathways contribute to the development of cancer. In particular it will provide insights into the regulation, of the DNA replication protein PrimPol by the mitotic kinase PLK1, the mRNA processing protein Cip29, and will lead to studies where the functional significance of phosphorylation and other protein modifications such as Ubiquitylation and Sumoylation for the regulation of DNA replication and cell cycle control will be established. The initial beneficiaries of this knowledge will be other academic researchers working in the fields of cell cycle control, cancer biology and clinical oncologists.

In addition, this work results in the generation of a wide range of antibody, protein and DNA reagents as well as technological and methodological developments in *Xenopus* egg extract production and assays to analyse DNA Replication stress responses. I have made these reagents available to other researchers working in the field and will continue to make reagents generated under this project licence available to the research community. Therefore, in the first instance the main short term benefit will be the provision of reagents, expertise and the extension of knowledge in an important area of biology which will be of interest to a large research community as well as being of interest to researchers in the pharmaceutical and biotech industries.

This advancement in scientific knowledge and the resulting research outputs are unlikely to be translated into advances to treatments that will benefit the public during the duration of the project. However, a greater understanding of the way DNA replication stress response pathways ultimately permit the continued survival of some cancers will provide an important contribution to understanding of how current cancer treatments achieve efficacy as well as identification and development of drugs that will kill cancer cells with greater specificity and this understanding will contribute to improvements in treatments for patients in the longer term.

Also, engagement with the public in publicising and explaining these outputs will also serve to increase public understanding of cancer, its causes and the recent improvements in treatments and prognoses which in turn is likely to have a beneficial effect on the number of people accessing healthcare advice regarding potential cancer symptoms.

How will you look to maximise the outputs of this work?

The impact of this work will be maximised through sharing data with other researchers as well as disseminating the research findings through publication in general/specialist interest international journals. Collaboration has been central to the success of my previous work in this area and such collaboration will ensue that the insight and knowledge generated by this application will continue to be the main route that our expertise in this non-human model system will be successfully exploited to further our understanding of human diseases such as cancer. Several long standing and successful collaborations have been established to bring the key expertise necessary together to maximise the impact of the work conducted and allow translation of the *in vitro* work into effective research using mammalian cells and new collaborations will be established should these be necessary to achieve the aims of the project.

Species and numbers of animals expected to be used

- Xenopus laevis: 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project will use egg extracts prepared from the unfertilised eggs of the South African amphibian Xenopus laevis. *Xenopus* are unique as they can be induced to lay eggs all year round and are therefore able to provide material for experiments as required. The eggs are large and contain the large stores of protein and mRNA to support many rounds of cell division with means that large amounts of material can be obtained from relatively few animals.

Typically, what will be done to an animal used in your project?

Laboratory bred male and female frogs are kept in a purpose built aquatic habitat in tanks containing 8- 10 animals each. A water treatment unit conditions and circulates water through the tanks. The environment of the habitat is constantly monitored to ensure optimal, temperature and water quality to keep the animals healthy and in good condition. The day before eggs are required, female frogs are induced to lay eggs by injecting a small quantity of HCG under the skin. Once egg laying is completed (18-36 hours) the animals are returned to the colony.

Male animals provide sperm to be used as a DNA template in DNA synthesis and nuclear formation experiments. Sperm production is also stimulated by injection of HCG. 5-8 days after injection, males are painlessly euthanized before the testes are removed and the sperm prepared for use in experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals display little or no distress during handling and the injection procedure itself. The instances of adverse effects caused by the injection (infection) are extremely 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 animal type)?

The severity of the procedure supporting this project is mild

What will happen to animals at the end of this project?

- Kept alive
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present it is not possible to reconstitute DNA damage response pathways from purified protein components. Therefore, in order to achieve the project objectives it is necessary to use material obtained from animals.

Which non-animal alternatives did you consider for use in this project?

Various invertebrate models, yeast, fruit fly as well as mammalian tissue culture cells

Why were they not suitable?

It is not possible to prepare cell extracts from invertebrate systems that posess the range of properties shown by egg extracts made from Xenopus eggs. As a result the ability of cell-free extracts from Xenopus to recapitulate cell cycle and DNA damage responses *in vitro* cannot be replicated with the same level of synchrony using eggs from invertebrate species or extracts from mammalian cultured cells. I have also considered other methods of obtaining eggs/sperm from Xenopus that do not involve regulated procedures; However, the quantities of eggs and sperm produced by these techniques are too low to provide sufficient material to achieve the objectives of the work associated with the project

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The animals themselves are not used in the experiments directly (animals provide biological material (eggs/sperm) for experimentation) so the number of animals is ultimately determined by the quantity of egg extract required to achieve the project objectives. With regard to female animals, although there can be some variability in the quantity and quality of the eggs laid by individual animals we keep records of the laying performance of each of the tanks in the colony and where available, these historical records along with our extensive past experience means that we can accurately estimate the number of animals required to generate any given volume of egg extract. The number of male animals required has been calculated on the basis of the quantity of sperm required to support experiments which has been estimated on the quantities used for similar work carried out over the past twenty years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The average quantity of eggs and thus volume of egg extract that can be produced from each tank of animals will be estimated. It is therefore possible to estimate the minimum number of procedures that will be necessary to produce sufficient egg extract for each experiment. Therefore planning experiments in advance and calculating the volume of egg extract required we are able to induce egg laying using the minimum number of animals necessary. In addition, identifying instances when previously frozen extract is suitable for use in experiments whenever possible will further reduce the number of animals/procedures required to achieve the projects objectives over the lifetime of the license. Injecting male animals with a small quantity of hCG (protocol 2) maximises the yield of sperm from each animal which reduces the number of animals used over the lifetime of the license. In addition, sperm obtained from male animals is stored frozen in small aliquots and only the minimum quantity of sperm required to support individual experiments is defrosted to keep wastage to a minimum and further reduce the number of animals required to support the experimental work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The number of regulated procedures (hormone injection) will be kept to a minimum by planning experiments in advance. The volume of egg extract required will be calculated in advance, and hence the minimum number of procedures required to provide sufficient egg extract to achieve the aims can be estimated. Egg production will also be maximized by ensuring that at least three months elapses before an animal is induced to lay again. Typically an animal may be induced to lay eggs 2-3 times each year. In all cases animals are inspected prior to undergoing the procedure to ensure they are healthy and in good condition. Although freshly made extract is generally preferable for most applications, in some cases previously frozen egg-extract can be used without compromising the experimental objectives.
Therefore the number of procedures required to achieve the objectives of the project will be further reduced by using frozen extract wherever possible. In the event of surplus eggs being laid then all eggs of suitable quality will be used to make extract and any surplus egg extract will be frozen and stored in liquid N_2 for use in subsequent experiments, thus reducing the number of procedures required to fulfil the aims of the project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use adult male and female *Xenopus laevis* to provide the source of biological material (sperm, unfertilised eggs). The production of eggs is induced by a sub-cutaneous injection of human chorionic gonadotropin (HCG). This injection causes minimal stress or pain to the animals. Eggs are laid 14-18 hours after injection. Female animals are returned to the colony after eggs have been laid and are not used again for the production of eggs for a minimum of 3 months to ensure the ovaries have sufficient time to repopulate with mature oocytes. To stimulate sperm production, male animals are also injected with HCG but 5-8 days after injection the males are anesthetized and euthanised prior to removal of the testes.

Why can’t you use animals that are less sentient?

The egg extract that is vital to the success of the work requires the production of high quality unfertilised eggs from the female *Xenopus* so only sexually mature adult female and male animals can be used. *Xenopus* is one of the few amphibians that can be induced to lay eggs in response to exposure to hormone and the characteristics of the eggs (size, type and quantity of biological material obtainable) cannot be replicated using less sentient organisms. On injection of HCG it takes 14-18 hours for egg laying to occur which would not be possible if the animal had been terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Although animals do not show significant distress during general handling and when undergoing the regulated procedure, handling is always kept to a minimum. Animals having undergone the procedure are kept in individual tanks which are placed in a dark quiet location overnight while egg laying is taking place. Once egg laying has been completed and the eggs have been harvested the animals remain in their tanks for 24 hours until any residual egg laying has been completed. Animals are then returned to the colony. There is evidence that feeding of animals is associated with a transient rise in the bacterial content of the tank water. As animals having undergone egg laying may be less inclined to eat and be more vulnerable to infection the animals are not fed for 24 hours after having laid eggs, before being returned to the usual feeding regime of the colony. The animals are routinely fed three times a week. Published guidance on the care and husbandry of *Xenopus* (RSPCA 2005) indicates that in the wild *Xenopus laevis* can survive periods of starvation of several months so we do not feel that the animals will be unduly distressed by withholding food for this short period after they have been induced to lay eggs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The animals themselves are not the subject of the experiments proposed in this project. The role of the animals is to provide the biological material (males-sperm, females-eggs) that are used in the experiments. The care and husbandry of the animals will be carried out using the benefit of over 30 years of experience of handling and maintaining these animals successfully to provide the biological material to support laboratory research. This experience will be coupled with reference to published guidance on the care and husbandry of *Xenopus* from organisations such as LASA and the RSPCA as well as published home office guidance on the care and housing of amphibian species.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are part of a worldwide collaborative network of research groups that use *Xenopus laevis* and *Xenopus tropicalis* for laboratory research. This network is active in disseminating information and providing resources regarding improvements in the care and husbandry of *Xenopus* species in a laboratory context. In addition we work with the manufacturers of the Xenoplus aquatic habitat systems to keep up to date with developments and refinements to the equipment used to house amphibians.

83. The Role of Central Sympathetic Control Neurones

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Autonomic control, Cardiovascular control, Physiology, Pharmacology

Animal types	Life stages
Mice	adult, juvenile, aged, embryo, neonate, pregnant
Rats	juvenile, adult, aged, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We and other scientists have identified a family of nerves in the brain that can control the heart and blood vessels. When these nerves are active they increase the blood pressure and heartrate.

Evidence suggests that excessive activity of these nerves could contribute to cardiovascular disease, especially in older people. We do not know what normal function these nerves have though. Scientists have several ideas including the suggestion that they could be the nerves that cause sharp and dangerous increases in heart rate during a sudden shock or fright. The central objectives of this project are to find out what the function of these nerves really is in a normal animal, and how we can modify their activity with drugs to prevent death and disease in people and domestic pets.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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, firstly itwill provide new avenues for targeting cardiovascular disease and secondly, it will teach us more about how the cardiovascular system is controlled and allow improvements to computer models of this which may, ultimately, reduce the need for animal experiments.

What outputs do you think you will see at the end of this project?

Information:

We will learn about the native control mechanisms of cardiovascular neurones in the paraventricular nucleus of the hypothalamus (PVN). We will learn what the role of these neurones is in the overall pattern of cardiovascular control.

Publications:

We will publish these data in high quality international journals as we have done with the previous experimental data.

Products:

We do not envisage products resulting from this work, but we have identified candidates (both in terms of specific mechanisms and proteins that may the foundation of novel pharmacological or biological products in the future for control of hypertension or heart disease.

Who or what will benefit from these outputs, and how?

Two clear beneficiaries are Academics seeking to understand how subtle and complex cardiovascular mechanisms operate and Industry who will find useful targets for novel medicines; pharmacological agents and biologics for heart disease and hypertension.

How will you look to maximise the outputs of this work?

Collaboration

Via publication and presentations we seek to engage with other physiologists as well as our medical colleagues. We work particularly closely with the Hospital ITUs physicians who are anxious for novel medicines to control cardiovascular system in crises situations. There are a range of current medications, but in these crisis situations there are always off target effects that need to be navigated.

Dissemination
We hold regular public engagement events to discuss the cardiovascular system. This is with schools, children and adults.

Unsuccess

We plan to publish all data including negative, but together with presentations where we discuss "fails" we hope to prevent wastage of time and animal lives in the future.

Species and numbers of animals expected to be used

- Mice: 300
- Rats: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our objectives are to understand the fine cellular and protein level mechanisms of control of the cardiovascular system by the brain. This is fundamental scientific knowledge and will inform future development of medicines that control and protect against cardiovascular diseases in humans and domestic animals. In humans, cardiovascular disease kills approaching 100,000-200,000 people per year working out to be approximately 500 people per day. To study this in detail one needs an analogous mammalian species where the main areas of the brain and the cardiovascular system fundamentally similar. This is the case with most juvenile and adult mammals, not necessarily other animal classes. However, mice and rats have long been studied in fine detail and so we have a vast database of protein and physiological data on their make-up making further scientific advancement realistic. Furthermore, as small and social animals they are easy to keep, look after and work with in comfortable home cages without the stress or discomfort that would be seen in a larger animal, such as a cat, dog or pig.

Typically, what will be done to an animal used in your project?

The primary objectives in this proposal are to investigate mechanisms by challenging animals with either drugs, biologics or stimuli and measure the resulting change in the heart rate and blood pressure etc. Most drug/biologic challenges are by simple injection, but some require the agent to be implanted in the animal requiring a surgical procedure under general anaesthetic. In terms of recording, we have 3 methods; an entirely non-invasive method working on the sample principles as a human arm cuff blood pressure meter (plethysmography), telemetry where transmitters are surgically implanted under the skin, or by arterial cannulation, a technique done entirely under anaesthetic. Each of these methods has strengths and weaknesses. Non-invasive requires no surgery, but can only record for a few seconds at a time (and a limited range of parameters can be measured), cannulation can record for 30 minutes and record all parameters within that time, but the animals are asleep and therefore recorded parameters have to be interpreted with that in mind. Finally telemetry is emerging as the optimal technique for many experiments since the animals can move about and behave normally and data can be collected for a few weeks without any evident discomfort or stress to the animals. Telemetry, however, fails to record some of the parameters that can be recorded with cannulation.

What are the expected impacts and/or adverse effects for the animals during your project?

We are confident that the drugs/biologics will all be harmless, but we will monitor carefully and in the unlikely event that we detected (untreatable) adverse effects we would terminate the experiment.

In terms of some of the surgical preparation, this is always done under general anaesthetic with pain killers. We use the same medicines that would be used for surgery in a veterinary practise, and this is verified by our veterinarian. If analogous surgery was done on a human for medical reason, much of it would be done under a local anaesthetic, but with animals it is important that they sleep through, so that they neither move nor become frightened. It is likely that there is some discomfort when the animals first wake, but this should be mild. We watch carefully and monitor continually as animals wake up to be sure they are not in distress. Typically animals are socialising and grooming (showing normal behaviour), but if that was not the case we would seek veterinary support. If the animals showed distress that could not be quickly rectified by ourselves or the veterinarian, we would put the animal to sleep permanently whether or not this was directly due to the surgery, experiment or anything 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 animal type)?

The official classification we have given for this work is "moderate", this is because there is general anaesthetic use, but the procedures should be otherwise harmless. No animals should be in distress at any stage, but general anaesthetic is not trivial for any animal. Some of our procedures involve "stress" tests; these are calculated to be mild however. We monitor heart rate and blood pressure during these and the level of stress would be equivalent to a sports event or exam, certainly not terror; furthermore it would be for a period of seconds or minutes and not chronic. Brief stress in a healthy animal is thought to be harmless, although in the elderly people or those with heart disease it can be harmful. We do not anticipate any lasting harm to these animals from our stress tests. Chronic stress, even in otherwise healthy animals, from guinea pig to humans will cause physiological damage and we will NOT approach those levels in this work.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project's central purpose is to understand the cellular mechansims by which the brain controls the cardiovascular system. It is a far more complex system than often thought and the only way to do this in the level of detail we propose is to include study of whole animals with a functioning heart and brain.

Which non-animal alternatives did you consider for use in this project?

We considered three primary alternatives to animals here; human studies, cell culture and mathematical models.

Why were they not suitable?

Human studies. In order to test mechanisms we need to do test drugs and procedures which, whilst we believe them to be harmless, could potentially have a low frequency of unexpected negative consequences. In a rodent study, we have the advantage that if this occurs we can humainly terminate the experiment and put the animal to sleep. Clearly with humans we could not do this and so we cannot get anywhere near the level of mechanistic insight we can get using animal studies.

Cell culture. We do indeed use cell culture wherever possible, but whilst we can study some aspects of cardiovascular control we cannot test how specific neurones effect the cardiovascular system from this.

Mathematical models. Again, we use these where possible. We have built models that recapitulate some aspects of the system and these are scientifically interesting but have too many knowledge gaps to allow development of novel medicines or understanding yet. We hope this project will go some way to address 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?

We determined the number of animals we would need using extensive analyses of the scientific literature followed by mathematical modelling.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We investigated several different numerical scenarios to devise the optimal number animals to use. Too few would mean the animals were wasted and too many would by definition be a waste. The approach we used was mathematical simulation using modern high performance computers and the
common mathematical programming language Python. In essence this includes using factorial designs such that a number of factors (n) can be assessed without need for n times as many experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As described previously we use mathematical modelling to optimise the number of experiments necessary. One key means we are reducing animal numbers is by constructing in silico models of both central processing neurones, and of the cardiovascular system as a whole. We impute data from the Public data sharing databases where available so we do not need to replicate those experiments, and we share our data in public repositories too. Where at all possible, we share tissue between groups.

We are sometimes able to collect brain tissue (just one example) from animals that are being euthanised anyway and this greatly reduces the number of animals that will practically be euthanised for this project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are studying the brain, cardiovascular system interface and it requires and animal to do this.

Where possible, we will use the least invasive method (in our case the tail cuff blood pressure system) and in experiments where more detailed cardiovascular parameters are necessary, we will ensure animals are adequately anesthetised and pain free.

Why can’t you use animals that are less sentient?

Our models need to be analogous to humans domestic pets; mammals in other words with fully developed brains and cardiovascular system such as heart and lungs. Invertebrates have similar neurones, but not similar cardiovascular systems. Non mammals do not have the same control areas of the brain. Neonates and embryos have very different cardiovascular systems.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We acclimatise animals before use with regular human interaction, we monitor the animals ourselves, and have additional help from the animal house together with regular inspections by a full time veterinarian. We use the latest veterinary pain killers, sedatives and anaesthetics where appropriate to improve animal welfare. We also use environmental enrichment in high quality home cages. Before using the non-invasive equipment, animals are acclimatised to this too,
Furthermore, we can improve data quality and thus reduce animal numbers by ensuring we have healthy stress-free animals, therefore, animals are housed comfortably within clean cages and handled regularly by expert staff (so they become used to humans). The animals have spacious cages and fresh air and unlimited clean water. Bedding is changed regularly and their health is monitored by an experienced veterinarian.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the Animals in Science Regulation Unit (ASRU) Guidance and regulatory advice and adhere to the guidance on the operation of the Animals (Scientific Procedures) Act 1986 at all times.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We read the 3Rs scientific literature, follow NC3Rs press releases and materials and attend 3Rs sessions and seminars.

84. Interventions in pregnancy: effects of obesity on maternal and offspring health

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

pregnancy, type 2 diabetes, cardiovascular disease, interventions, programming

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess the possible benefits and risks of lifestyle changes and interventions (e.g., taking of medications or exercise) in obese females before and during pregnancy on both the immediate and long-term health of the mother and her 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?

In western populations such as the UK, 50% of all women of child-bearing age are classified as overweight or obese. The National Institute for Health and Care Excellence (NICE) highlights the negative health risks of this on both the mother and her offspring in the womb and beyond.
One such complication in pregnancy is diabetes. If making changes to the woman's diet

and lifestyle is unsuccessful in improving the condition, metformin (a drug in tablet form) is the next step. Whilst metformin is very successful in treating diabetes in the mother, little is known about its long-term effects on the offspring. Therefore, it is vital to investigate this important gap in our scientific understanding and assess if the benefits of metformin on the mother, outweigh any possible risks to her offspring.

We also aim to identify, assess and understand other types of interventions that could prove beneficial in improving the health of women and their children for example, exercise and the taking of antioxidants. Antioxidants are substances that slow down or prevent damage to cells in the body.

What outputs do you think you will see at the end of this project?

We intend to publish all findings (positive or negative) as research articles which will have been checked by independent scientists in the field. We will also present our findings at scientific meetings in the UK and overseas.

Who or what will benefit from these outputs, and how?

In the short term, it is expected that our published data will provide understanding of the ways in which interventions influence (positively or negatively) the health of the mother and her offspring. These data will inform health professionals (e.g., nurses, doctors, health visitors) about the effects of lifestyle choices and medicines in pregnancy for obese women. Data produced from these outputs will be used within future grants to fund further research into this area. These grants may include those for basic scientific research and/or clinical trials.

In the mid to longer term, these studies will result in collaborations, including clinical research groups and may benefit those such as the pharmaceutical industry. Our basic research will allow a directed approach to assess our findings in human trials which would further knowledge transfer and improve health.

How will you look to maximise the outputs of this work?

We expect to publish in high quality journals and in order to do this our work will be assessed by independent scientists (those not working with our group) to make sure that the science is of excellent quality prior to publication.

Negative data (results that do not provide the expected outcome/result, or in the case of comparing two experimental groups, there are no differences for the measured outcome) usually forms part of all scientific publications. Therefore, negative data will not be dismissed but will be presented at scientific conferences and within scientific papers as is routine.

We will also engage with and disseminate information to other stakeholders including our funding bodies and the public. For example, in the past we have been involved directly with the funders (e.g., British Heart Foundation) by engaging volunteers and donors in events that allow them to observe / carry out the types of experiments we conduct as part of our research. These opportunities are likely to present themselves again in the future.

We will also continue to engage with schoolchildren and the general public through events such as science festivals. We are also very active on Twitter, with over 1200 followers which allows us to continue to disseminate the results of our work to a wider audience.

Species and numbers of animals expected to be used

- Mice: 16,800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have been using mice to generate our model of obesity in pregnancy for approximately 12 years. During this time, we have consistently shown that this model has distinct similarities to many aspects of an obese human pregnancy. For example, the obese mice gain weight between their first and second pregnancies and they show signs of diabetes in pregnancy. We have also shown that similarly to humans, offspring born to obese mothers have a higher risk of disease (e.g., heart disease) in later life.

Mice allow us to study organs and tissues that would be either impossible or unethical in humans. For example, in order to understand what is going on in an organ at the level of the cell, we need to collect organs from the mother and/or offspring for us to perform our laboratory analyses. We also need to study effects of the mothers' diet on her offspring across their life course which are only feasible to animals with short lifespans such as mice.

Life stages required

Female mice before, during and afterpregnancy

- As mice are only pregnant for 21 days this allows us to perform experiments in a feasible time- scale which would be impossible in humans where a pregnancy is much longer at 9 months.

- As our research aims to understand the impact of different environments during pregnancy, it is essential to study females before, during and after their pregnancy.

- *Developing unborn offspring (embryo/fetus) at multiple stages of development (during pregnancy).*

- In order to understand how various maternal environments in pregnancy affect the growth and development of the unborn offspring (fetus) and the placenta (organ that allows the exchange of nutrients and oxygen from the mother to her unborn offspring), it is necessary for us to use tissues from the unborn offspring at different stages of development. Collection of such tissues would be impossible in humans.

Offspring at various ages once they have been separated from their mother.

- We need to use offspring at multiple ages exposed to one of four different pregnancy environments (healthy lean pregnancy, obese pregnancy and lean/obese pregnancy with intervention treatments such as drug treatment or exercise). We aim to study the impact of these different pregnancy environments on the future development of diseases such as type 2 diabetes and heart disease.

- Mouse models are critical for this research as they have a short lifespan (approximately 2 years), which allows us to study these diseases which develop with age. If we were to study the same effects in humans, we would have to follow the mother and child across an entire generation (30- 50 years).

- This mouse model will provide understanding of the consequences of obesity in mouse pregnancy and the risk *vs.* benefit of such strategies to mother and offspring. This information will provide evidence to form a more targeted approach in studying the immediate and longer term impacts of obesity in human pregnancy.

Typically, what will be done to an animal used in your project?

Female mice (who will become mothers) will be weaned onto either a healthy diet or a diet high in fat and sugar and they will remain on these diets for their lifetime. This will generate our two main experimental groups, (1) control females (healthy pregnancy), (2) obese females. Both groups of mice will then be mated for a first pregnancy to ensure that they are fertile and will rear their young successfully. After this first pregnancy, we check that the mice fed the diet high in fat and sugar have gained excess weight and fat mass compared to the lean healthy mice before mating both groups for a second pregnancy. The second pregnancy is known as our experimental pregnancy.

Females who become pregnant for their second time, with or without any interventions (e.g., drug treatments or exercise), will be allocated to one of three possible outcomes.

To study the effects of an obese or healthy pregnancy on the health of the mother and her unborn offspring.

To allow the female to give birth and rear her offspring. Her offspring will then be studied.

To study the direct effects for example of hormones, on the development of disease in the offspring. This outcome requires the surgical procedure, listed below.

Note, the interventions can be implemented either before pregnancy, during pregnancy, during lactation (period where the mother is suckling her young) or a combination of all three.

Measurements in females, before and during pregnancy.

They will have their blood pressure measured using a non-invasive method that is similar to the way humans have their blood pressure taken, except it is done on their tail rather than on the arm. Typically this will be done on 6 occasions and maximally on no more than 8 occasions during the life course of the female.

To measure the degree of obesity, mice will be placed (still conscious) in a tube to have their fat and lean (muscle) mass measured inside an imaging machine such as an echo-MRI. This is like an MRI machine used by humans, but just on a much smaller scale. This is not invasive and usually takes less than 5 minutes. Typically this will be done on 7 occasions and maximally on no more than 10 occasions across the life course of the female.

To assess the heart health of the female and the health of her unborn offspring, we may choose to perform a non-invasive ultrasound scan. To prevent the animal from getting

distressed whilst being handled and held for an awake procedure, we place them under a light anaesthesia (which is inhaled) and would typically last no longer than 2 hours. Mice will be gently awoken after the procedure and monitored closely afterwards. Typically this will be done on 1 occasion and maximally on no more than 3 occasions across the life course of the female.

Mice are usually housed together, but during and after pregnancy our mice are housed alone. We do this so that we can keep a closer eye on the mother (including her daily food intake) and her health during pregnancy. For offspring studies where females are required to litter, we will be able to check on her pups regularly and be certain on who is the mother of the individual pups in the cage.

In human pregnancies, mothers suspected of having diabetes will have a glucose tolerance test where they are given a sugary drink (glucose) and their blood glucose measured at multiple intervals over a 2-to-3-hour period. We will perform the same test in the mice but instead of a sugary drink, glucose will be injected into the peritoneal cavity (lower half of the abdomen) and glucose measured in tail blood at timed intervals over a 2-to-3-hour period. Typically this will be done on 2 occasions and maximally on no more than 4 occasions during the life course of the female.

In order for us to assess the health of the female, we may take small quantities of blood from the tail which would allow us to measure important read outs of health such as blood sugar levels, insulin and cholesterol. Typically this will be done on 3 occasions and maximally on no more than 6 occasions during the life course of the female.

Some pregnant mice will receive an injection of dye (between day 12.5 and day 18.5 of pregnancy, that corresponds to mid to late pregnancy), which will allow us to measure how quickly embryonic cells divide. This injection is delivered quickly into the abdomen. The mother is then left to give birth and we are able to assess the results of the injection of dye in the offspring. This will typically be done only on one occasion and no more than two occasions.

In some pregnant mice a different dye will be injected (between day 12.5 and day 18.5 of pregnancy, that corresponds to mid to late pregnancy) that will allow us to determine if there is poor oxygen delivery to the unborn offspring and their placentas. This injection is delivered quickly into the abdomen and the mice are killed humanely up to 180 minutes later.

For those females that have not been used for point 8, at the end of the study we may perform ultrasound imaging to assess the health of the heart, placenta and fetuses in addition to their response following injection of substances that can speed up or slow down the heart. We will do this under inhaled anaesthesia and once the procedure is finished, the mother and her embryos will be humanely killed whilst still under anaesthetic.

At the chosen end time-point within pregnancy (for those mothers not entering at point 7, 8 and 9), the mother and her embryos will be humanely killed and all tissues will be collected following the confirmation of death and stored for future laboratory analyses. These tissues will be available to our group and collaborators upon request.

Females that are required to litter to allow for the generation of offspring will suckle their pups until weaning (when her pups are 21 days old). After this she may undergo a glucose tolerance test (as described in point 5) prior to her being humanely killed and her tissues collected and stored for laboratory analyses. Her offspring will be housed together

grouped by sex, and fed either a standard (healthy) or obese diet and will undergo procedures listed below, under the heading 'Offspring'.

Surgery in pregnancy

A small number of females will undergo a surgical procedure during pregnancy. This procedure would be performed under general anaesthetic with all the surgical precautions taken pre-, during- and post- surgery. The surgery time is expected to be short (approximately 30 minutes in duration, from sedation to awakening). During surgery, a small incision in the abdomen would be made to expose the uterus containing the fetuses. At this point a very small volume of liquid containing a substance (for example a hormone) will be injected either into the amniotic sac where the fetus is contained or, into the fetal brain. Following injection, the incision would be closed with stitches or staples and the mouse allowed to regain consciousness by removing the anaesthesia. She will receive appropriate pain medication and will be observed by the scientist performing the procedure and by animal technicians. She will then be observed daily to ensure she was in no pain and that the wound was healing as it should. She will be monitored daily to ensure she has a normal birth.

Once recovered these females will be left to litter and bring up their offspring up until weaning (when the pups are 21 days old). At this point the mother may undergo a glucose tolerance test (as described in point 5) prior to her being humanely killed and her tissues collected and stored for laboratory analyses. Her offspring will be housed together grouped by sex and fed either a standard healthy diet or obese diet and will undergo such procedures listed below, under the heading 'Offspring'.

Offspring

A lot of the non-surgical methods used in the 'Measurements in females, before and during pregnancy' section will also be carried out in the offspring as we try to gather an understanding around their heart health and their risk of developing obesity and diabetes.

Assessing fat and lean mass by the method detailed in point 2 above. Typically this will be done on 7 occasions and maximally on no more than 10 occasions across the life course of the animal.

Measuring blood pressure non-invasively on multiple occasions throughout their lifetime by the method detailed in point 1 above. Typically this will be done on 6 occasions and maximally on no more than 8 occasions during the life course of the animal.

Glucose or insulin tolerance tests which require injection of either glucose or insulin by the method detailed in point 5 above. Typically this will be done on 2 occasions and maximally on no more than 4 occasions during the life course ofthe animal.

Blood sampling by the method detailed in point 6 above. Typically this will be done on 3 occasions and maximally on no more than 6 occasions during the life course of the animal.

We have the capability of measuring offspring food intake by weighing food daily within the animal's home cage. We may do this by housing the offspring on their own (single housing) so there is no competition between multiple mice for food. This will provide us with an accurate result on an individual mouse’s drive to eat. Typically this will be done on 3 occasions and maximally on no more than 6 occasions during the life course ofthe animal.

Offspring heart health (using echocardiography) will be assessed using a non-invasive ultrasound scan which will be performed under inhalation anaesthesia (and the mouse will remain anaesthetised throughout the procedure). Typically, the procedure takes no longer than one hour. Animals will be awoken at the end of this procedure. Typically this will be done on 3 occasions and maximally on no more than 6 occasions during the life course of the animal.

Similar to the dams' section, we will also assess offspring heart health in response to a range of drugs that for example, can speed up or slow down the heart rate. Animals will be sedated for the procedure and be killed humanely whilst still under anaesthesia and tissues collected for storage within our lab tissue bank.

To further understand the risk of the offspring developing diabetes, we will perform a procedure called a euglycaemic clamp. In this procedure the offspring will be fasted overnight, and their blood sugar (glucose) levels recorded. Blood glucose levels are measured from a small drop of blood from the mouse's tail. The mouse will then be anaesthetised (and will remain anaesthetised throughout the procedure) and a known quantity of insulin (hormone) will be injected into the abdomen. This will lower the blood glucose levels. Using a cannula inserted into the tail vein, glucose will then be slowly administered to increase and then maintain blood glucose levels at its starting value. After this procedure the mouse will either (a) killed by an overdose of anaesthetic (b) injected with a substance to kill the animal while preserving organ and tissue structure while still under surgical anaesthesia.

Offspring that have not come from point 6 and 7 will be humanely killed at the end of the experiment and their tissues collected and stored for future laboratory analyses for us by our group or collaborators upon request.

A small proportion of surplus offspring (that would otherwise not be used/culled for statistical reasons) and that have only had minimal phenotyping e.g. weighing, body composition or non- invasive blood pressure measurements may be transferred to other collaborative projects for further specialist techniques for which we do not have expertise, i.e. feeding behaviour, autonomous feed preference. This is important to ensure we maintain good 3Rs practice and would avoid the need for more pregnant mice and offspring to be generated.

Any surplus genetically altered mice that have not undergone any regulated procedure will also be made available to other users to maintain good 3Rs practice.

What are the expected impacts and/or adverse effects for the animals during your project?

As the large majority of work is non-invasive there are very few procedures within this license that would be expected to cause any impact or adverse effect.

However one procedure that would be expected to cause adverse effects would be surgery. The surgical procedure will be carried out under anaesthesia and pain will be alleviated through the use of pain relieving medication. We have kept the surgical procedure short to reduce any complications during anaesthesia. Complications surrounding wound healing (where the animal was cut open) are expected to be rare, but where these cases occur we will ensure their pain is adequately controlled using pain relief. In the rare cases where the wound re-opens we will place the animal back under a

short anaesthesia to repair the wound, we will only repair the wound once. If neither of these improve the outcome, the animal would be humanely killed. Post-surgery monitoring will be carried out by the experimenter and animal technicians multiple times per day in the first few days post-surgery and all mice will be provided with soft bedding to provide optimal conditions for quick / uncomplicated healing and housed together where possible.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice

Mild = 98%

Moderate = 2%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Modelling obesity in humans is highly complex as there are a number of physiological alterations that are impossible to model in cell culture systems. A cell culture system involves growing cells in a dish within the laboratory under tightly controlled conditions. The growth of these cells can be monitored and expanded to accommodate the experiment required at the time.

As we are studying long-term health impacts of obesity in pregnancy on the offspring, the short pregnancy (21 days) and lifespan (approximately 2 years) of the mouse model means that we can study disease progression within a short time frame (i.e., for duration of a project). Typically, in humans this could take half a century to study.

Which non-animal alternatives did you consider for use in this project?

Based on findings generated from our previous project license we were able to replace some of our animal work and use cell lines generated from humans and animals to further investigate findings from our animal models in cell culture (where cells are grown in a small dish in the laboratory to increase the number of cells available to do experiments). Cell lines from animal models are still classed as a replacement as they have been immortalised, therefore no further use of animals is required. We will continue to do this as part of this PPL.

Why were they not suitable?

These non-animal alternatives are not suitable for total replacement of animals within our project as we still cannot model pregnancy or the complexity of obesity in a cell culture system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based the estimated number of mice on the numbers and findings from our previous and current project licenses and our Home Office returns which we return annually.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Multiple users within our research group will coordinate their projects to work on different organ systems while using the same mice. This will not only allows us to gain whole body information on a single mouse, but significantly reduce the number of animals used. This method will also improve data quality as the results from different organ systems can be compared within the same mouse.

To further reduce the numbers of animal used and to replicate what happens clinically whereby only obese women would be offered interventions in pregnancy, we usually only implement intervention strategies in obese females.

We would also utilise where possible banked tissues and cell lines from wild-type and genetically modified animals in the first instance prior to generating any new cohorts.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As we only require the female offspring (from our in-house stock breeders) for our pregnancy experiments, the male offspring will be made available to other users in the facility. Ex-stock breeders will also be made available to the facility for other users. Furthermore, offspring born from the first pregnancy will be offered to other users within the facility or made available for use by members of the lab for example for use in optimisation of molecular analyses.

Being a pregnancy model, we study both the mother (during/after pregnancy) and her offspring (from birth to adulthood) to maximise the measurements and to integrate the outcomes observed for both mother and offspring.

Furthermore, we have an extensive tissue bank of samples that can be utilised prior to generating new animal cohorts. This tissue bank is available to both our group and external collaborators on request.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will only be using mice within this project license. The majority of our methods are non-invasive and these were chosen in order to not only mimic what measurements can be (and are) performed during a human pregnancy but also to minimise any effects on the mice. Some of these methods include the measurement of blood pressure, fat and lean mass, metabolic and heart function as well as assessing placental function and the health of the unborn offspring. All of these measurements cause only very minor or no pain or lasting harm.

Only one of the methods proposed will involve surgery under anaesthesia (sedation). Surgery time is short and will be expected to last approximately 30 minutes. Once awoken the mouse will receive pain relieving medication and constant monitoring by the experimenter and animal technicians. To reduce the procedural and anaesthetic time for the female and her unborn offspring, we have devised a short protocol with a very specific aim. This will ensure that the pregnant female makes a quicker recovery.

Why can’t you use animals that are less sentient?

For a significant number of our protocols we are able to use animals that are less sentient (able to feel things).

Collection of unborn offspring and their tissues.

Culling of pups to standardise litter size to ensure all pups get a good amount of nutrition from the mother.

Death under terminal anaesthesia of pregnant females following non-recovery echocardiography / ultrasound.

Death under terminal anaesthesia of offspring following a euglycaemic clamp.

Death by perfusion fixation under terminal anaesthesia. Perfusion fixation ensures that organs and tissues are preserved. This process starts by pumping a salt-containing liquid through the blood vessels, followed by a liquid fixative (to ensure that tissue structure is maintained). Tissues will then be stored to be used later.

However, for our other work it is not possible to use animals that are less sentient. As we are modelling obesity in pregnancy and aim to assess the long-term health of the offspring, we need to use animals that are sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Based on our previous experience we have been able to refine some of the procedures to minimise harm. Surgery will be performed under aseptic conditions (set of procedures that protect the mouse from infection during surgery), anaesthesia (state of unconsciousness/sleep) and analgesia (pain relief) will be provided immediately, for as long as necessary. There will also be increased monitoring of these animals which will include measurement of body weight and regular checks on wound healing. Any animals exceeding a certain level of weight loss will be humanely killed. Mice will also be provided with soft post-operative bedding, nesting materials, heat pads and mash food where necessary. Pain will be monitored, and pain relief provided in the form of oral medication (e.g., flavoured jelly, paste or milkshake liquid).

Once our experimental animals become pregnant, these females are often housed alone. The reason we do this is to increase the pregnancy monitoring to ensure the health of the dam and her unborn offspring. Single housing also allows us to collect individual data on the dam during pregnancy (e.g., food intake). To create an enriched environment for singly housed animals, enrichment (e.g., tubes, tunnels, houses, nesting materials) will always be provided.

We routinely acclimatise our mice to the blood pressure machine over a period of training. We will continue to do this within this license and for other settings where restraint or handling is required. A similar period of training is used for mice that will be exercised such that we start with lower speeds to allow them to become familiar with the equipment and then gradually increase the running speed over time. There is also a sponge at the back of the treadmill that encourage mice to run, rather than standing stationary. Mice will typically be put on the treadmill at the onset of the dark cycle mice to take into account their nocturnal nature. Mice that refuse to run on multiple occasions will be removed from exercise training.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There is a wealth of resources published on the NC3Rs webpages and this is our first port of call for guidance on best practice, in addition to following and adhering to the ARRIVE guidelines. We will also use and refer to the PREPARE guidelines when planning animal experiments. Further to this, especially for use in surgery, we will refer to guidance from the Laboratory Animal Science Association (LASA), especially LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.).

We also seek advice from our peers especially those who have successfully published similar studies in mice to gain from their experience.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain good contacts with the lead individuals (Named Persons) within our animal facility where we will be informed of any new developments or changes. We also have a 3Rs committee at the establishment and their minutes are available via our institution intranet. Our group will attend termly facility user committee meetings where new information is disseminated.

We will also aim to ensure that people working under this license are subscribed to the 3Rs newsletter from the National Centre for the 3Rs (NC3Rs). In addition to this we will continue to seek knowledge from the NC3Rs webpage (https://nc3rs.org.uk/resource-hubs), and 3Rs tools in-house and external resources such as the Laboratory Animal Science Association (LASA), RSPCA scientific group and Norecopa (https://norecopa.no/databases-guidelines).

In addition to this we are also aware of symposiums, talks, webinars and workshops all discussing the 3Rs. We will endeavour to stay on-top of advancements by attending these events as regularly as possible.

85. Breeding and maintenance of genetically modified and harmful mutant animals

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

breeding, maintenance, GA genetically altered, GAA genetically altered animals, HM harmful mutant

Animal types	Life stages
Mice	adult, pregnant, aged, neonate, juvenile, embryo
Rats	adult, pregnant, aged, juvenile, neonate, embryo
Zebra fish (Danio rerio)	neonate, embryo, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To breed and maintain and supply genetically altered (GA) or harmful mutant (HM) rodents and fish for use in bio-medical 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?

Genetically altered and harmful mutant animals are essential for addressing many scientific questions of bio-medical importance including understanding the function of specific genes and the role they play in disease. The work conducted under this licence will enable the breeding of genetically altered and harmful mutant animal for use either in biomedical research studies conducted using tissues taken after the animal has been killed using a schedule 1 technique or in studies conducted under other project licences. This service licence will enable breeding programmes at the establishment to be optimised

and thereby minimise animal usage in line with 3Rs expectation.

What outputs do you think you will see at the end of this project?

Genetically altered and harmful mutant animals suitable for bio-medical research.

Who or what will benefit from these outputs, and how?

The primary benefits of the work conducted under this service licence will be to enable the breeding programmes at the establishment to be optimised to minimise animal usage, in line with the expectations of the 3Rs. The programme of work will benefit scientists undertaking studies performed using tissues obtained after the animal has been killed by a schedule 1 technique, or scientists conducting studies under the authority of PPL with authority to use genetically altered or harmful mutant of the type being bred.

How will you look to maximise the outputs of this work?

A tissue sharing scheme is in-place to ensure the maximum use of the animals bred under this licence.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 100
- Zebra fish (Danio rerio): 25000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most common and least sentient of the mammalian species used in scientific research. The ease with which their genetic make-up can be altered, the wide availability of mouse specific reagents, the extensive range of established disease models and the fact that they share many common physiological processes with humans makes them the species of choice for many bio-medical research projects.

Rats are the species of choice for studies that require an animal that is either physically larger or has a greater ability to learn and problem solve than mice. The genetic manipulation of rats is far more difficult than mice, however several important genetically altered and harmful mutant rat lines exist that are of great value to researchers working on important diseases such as hypertension.

Zebrafish are the most common non-mammalian species used in scientific research and the second most commonly used animal under the Animals Scientific Procedures Act. The ease with which their genetic make-up can be altered and the translucent nature of their bodies, which enables many biological processes to be visualised, make them the species of choice for many basic research projects including studies into joint development and tissue healing.

The animals used under this licence will be adults, as only adult animals are able to breed. The offspring produced may be used at any stage in development, depending upon the scientific need.

Typically, what will be done to an animal used in your project?

Selected genetically altered or harmful mutant animals will be paired up and allowed to breed naturally.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals used or generated will suffer no harmful effects. A small proportion (less than 5%) may carry genetic alterations or mutations that could result in the development of disease. However, these will be carefully monitored against assessment criteria that ensure adverse effects are detected at an early stage. Any animal showing adverse effect will be humanely killed to prevent undue suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

sub-threshold 95%, mild 5%.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of the outlined work is to generate animals for use in scientific research studies performed on tissues collected after killing. A small proportion of animals may also be transferred to other PPL's for use under other projects.

Which non-animal alternatives did you consider for use in this project?

The use of non-animal alternatives are not applicable to this project.
Why were they not suitable?

There are no non-animal means of generating the complex tissues that compose body systems, such as the blood vascular, renal, immune or nervous 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?

The estimated number is based on usage over the previous five year period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All requests for breeding under this project must undergo independent ethical review, which requires the applicant to justify the need for the work, the choice of animal species and to provide the statistical methodology used to estimate the number of animals needed to meet the study aim. Animal breeding will comply with the recommendations outlined in the Home Office document 'Efficient breeding of GAA'.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The breeding program will be optimised and carefully monitored to ensure that, as far as possible, only the minimum number of animals required to meet the scientific need are bred.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals carrying genetic alterations or harmful mutations will be paired and bred naturally.

Why can’t you use animals that are less sentient?

All projects using animals generated under this project undergo independent ethical review, which requires the applicant to justify their choice of animal species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals used in this project will only experience natural breeding. Animals will be handled using refined methods e.g. tube handling or cupping and will be provided with environmental enrichment in the form of bedding, tubes or hutches and wood sticks for gnawing. All animals will be carefully monitored for signs of impaired wellbeing. Animals

with genetic alterations or mutations known to compromise their wellbeing will be monitored for the associated indicative signs and killed if they either develop these or show any overt signs of suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Breeding programs will be managed in line with guidance documents issued by the NC3Rs, Laboratory Animal Science Association, the Institute of Animal Technology and in compliance with the Home Office document 'Efficient breeding of GAA'.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will continue to meet regularly with our NC3Rs representative and to partake in 3Rs events. I am also a member of the Institute of Animal Technology who provide regular updates on 3Rs matters.

86. Assessing and improving welfare in dairy cattle and calves

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

animal welfare, animal health, preference, disease detection, precision livestock farming

Animal types	Life stages
Cattle	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to improve the health and welfare of cattle by designing cubicles that better meet the needs of cattle and by finding new methods of detecting disease, particularly in calves.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Housing for dairy cows and calves has primarily been designed around the needs of the farmers, in terms of ease of access to animals, ease of cleaning and time-efficiency for staff. However, traditional housing designs do not fully take account of the natural behaviour of the animal. While research has shown that cows prefer to lie in open bedded areas, the majority of dairy farms in the UK use a cubicle system in the lying areas for adult cattle. These cubicles consist of metal frames, that outline a rectangular space for a

single cows to lie in. However, the frame greatly restricts the lying posture of the cows. Cows can only rest on their chests/belly with their legs tucked in and cannot lie flat on their sides. Cubicles are used as they are easy to keep clean compared to open bedded areas, thus reducing the risk of mastitis, which is why the are becoming the norm in the dairy sector. However, cow preference for open lying areas does suggest that it is the restriction to the lying posture that is one of the issues, and work needs to be done to determine whether the design of cubicles can be altered to provide cows with more opportunity to lie in more relaxed postures (and improved welfare). The aim of this part of the project is to determine how the design of cubicles can be improved to better promote cow welfare.

Additionally, we aim to assess the validity and practicality of new methods and monitoring technologies to detect the presence of disease in cows and calves earlier than is currently typical. Disease is a major cause of mortality and poor health in dairy cattle and particularly in calves. Mastitis and lameness are major causes of poor welfare in adult dairy cattle and have major impacts on production and welfare.
Respiratory disease and diarrhoea are particularly prevalent diseases in calves. Calf disease is estimated to cost the UK cattle industry over £60M p.a. and costs £43 per case of pneumonia (NADIS, 2015) and £58 per case of scour (CHAWG, 2014) per affected calf. As well as causing mortality and illness in the young calf, the damage caused by the disease has long-term effects on growth and performance of the animal across its lifetime. Early detection of disease is important, as it allows the individual to be treated before the effects become too damaging, and it prevents the spread of the disease to other animals. This will improve welfare, but will also reduce the use of anti-microbial drugs in farming. Better health in animals will reduce the overall greenhouse gas emissions associated with farming.

What outputs do you think you will see at the end of this project?

At the end of this project, we will have a better understanding of how to construct lying areas for cows that allow them the type of space and bedding material they need to achieve good quality rest and sleep. We will also have new methods of detecting disease in calves. This will involve understanding how behaviour and physiology changes as animals become unwell, and creating methods and technological solutions to detect these changes.

This information will be disseminated to farmers, veterinarians and other stakeholders through webinars, open days and knowledge transfer documents. The findings will also be published in scientific journals and presented at conferences. The findings will also contribute to the development of devices and computer algorithms that can detect disease.

Who or what will benefit from these outputs, and how?

The studies on the design of lying areas for adult cows will produce information on how to best design lying areas that optimise the welfare of cows in a production setting. This information can be used by farmers or farm advisors to promote animal welfare when building new cow housing facilities. The findings can be used by policy makers when writing codes of recommendation for new buildings. If best practice in housing design is applied across the industry, it can be expected to have a positive effect on cow welfare and improve the sustainability of UK dairy farming.

The development of novel methods of disease detection in calves will have benefits for a number of different parties. Firstly, farmers could use these methods to identify individual animals suffering from disease early in the course of the disease. This would allow those

animals to be treated earlier than is the current practice, and before the full adverse effects of the disease are manifested. This would reduce the impact of the disease on animal welfare and reduce the number of costly treatments required. Allowing all calves to grow to their full potential without disease checks will have a positive impact on farm profitability, and reduce overall greenhouse gas emissions at the farm level as slow- growing animals take longer to reach a productive state and are less productive overall. Secondly, early detection and treatment reduces the risk of spreading the infection to other individuals in the group and herd. This has obvious benefits for health and welfare, but there are also societal benefits, as early detection and treatment of disease reduces the need for 'blanket' treatments of groups of calves which contributes to antimicrobial resistance.

We expect that these findings will become available as the studies are completed (from 2024 onwards) and will be disseminated through articles in the agricultural press, presentations at Open Days and shows, and directly to farmers via interaction with farm advisors.

How will you look to maximise the outputs of this work?

The best way to maximise the benefits of this work is to get the information out to the stakeholders who can make the most use of it. As we are working with commercial livestock, farmers are the most important stakeholders to reach. The transfer of any new findings will occur through presentations at Open Days, agricultural shows and by engaging with farm advisors, who deal with farmers on a daily basis. Other stakeholders, such as policy makers and industry bodies will be reached through the production of press releases and the production of research summary documents. The broader scientific community will be reached through publishing in scientific journals.

Species and numbers of animals expected to be used

- Cattle: 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All of the issues that we wish to investigate are problems that directly affect dairy cows and calves, so the greatest experimental validity is achieved by carrying out the experiments directly on these animals. It will also mean that any findings can be directly used to improve the welfare of these animals.
Currently, no viable alternatives exist for the systems we wish to investigate.

We are using adult cattle to investigate the preferences for lying areas, as the type of lying area under study is only used with adult dairy cattle. We are using calves in the trials assessing methods to detect disease, as the diseases we are studying are of high prevalence in calves and very low prevalence in adult cattle.

Typically, what will be done to an animal used in your project?

In experiments assessing cow preferences for different types of lying area construction,

cows will be housed on their own in a pen for up to 10 days. Other cows will be housed in adjacent pens, with visual and physical contact possible, so that each cow is not entirely isolated.

In experiments assessing methods and technologies to assess disease, blood samples are taken to assess the concentrations of certain chemicals that indicate the presence or absence of disease. Up to 8 blood samples will be taken from each calf over a period of 6 weeks. Additionally, to monitor the health of the calves, the temperature of each calf will be assessed daily by inserting a thermometer into the rectum. Some of the monitoring technology that will be tested is fitted into an eartag which is fixed to the calf using an ear-punch, similar to the current identification eartags that it is mandatory to attach to the ears of calves.

What are the expected impacts and/or adverse effects for the animals during your project?

Cows housed in single pens show some anxiety for a short period (less than one hour) when they are first put into a pen on their own. However, as we always arrange the test pens so that other cows are visible in neighbouring pens, the cows settle down and are observed to eat and lie down in a relaxed manner soon after being moved.

The application of an eartag to a calf results in some initial pain (similar to ear-piercing in humans) but resolves over the next day. An experienced operator applies the tag. The site of the tag is checked for signs of inflammation or infection and treated if necessary.

Taking a blood sample from a calf involves the pin-prick pain of entry of the needle to the vein. However, this is short-lived. Experienced personnel will take the sample to minimise pain.

Taking the temperature of a calf every day using a thermometer inserted into the rectum carries the possibility of irritation to the rectum. However, lubricating gel will be used on the thermometer, and experienced staff will carry out the procedure. We have used this procedure on many calves previously, and no problems of this nature have been seen.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severity of:

- Housing adult cattle alone in a pen with access to neighbours for up to 10 days: mild
- Application of eartags using an ear-punch to calves: mild
- Blood sampling of calves: mild
- Daily temperature monitoring using a rectal thermometer: mild All animals on test will experience this expected severity level.

What will happen to animals at the end of this project?

- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

All of the issues that we wish to investigate are problems that directly affect dairy cows and calves, so the greatest experimental validity is achieved by carrying out the experiments directly on these animals. Currently, no viable alternatives exist for the systems we wish to investigate. There are no computer models or other model species available, but we will thoroughly investigate potential new ones that arise.

Which non-animal alternatives did you consider for use in this project?

There are currently no non-animal alternatives available for this type of study.

Why were they not suitable?

None available to date.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 advice will be sought from expert statistical consultants in the design of each experiment to get the best design that makes the most of the data collected. Information on the sample sizes used in this application are based on previous experiments that have balanced the need for sufficient animals to achieve reliable results with the need to avoid over-use of animals. In experiments in which we are testing methods to detect disease, the sample sizes are based on the known prevalence of disease in our experimental herd.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The opportunity to reduce the number of animals used in the experiments is taken into consideration in the design of all experiments. Animals will be used as their own controls wherever possible, with base- line measures made to reduce the variation in the data due to differences between groups and individuals (e.g. comparing data from a calf when it is sick and when it is healthy). Cross-over designs will be used so that all animals experience all treatments, rather than having different groups experiencing different treatments.

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 with a small number of animals will be used to establish methods and screen options such as sensor design. Pilot trials will allow us to establish the best possible

methods before moving to a full experiment with a larger number of animals.

Animals used on all experiments will be sourced from our own farms so that they have had the same rearing and feeding systems throughout life and are of the same genetic stock. This will reduce the 'noise' in the data that is due to previous history and genetics. All animals used to be checked to confirm health, except in the studies that aim to develop methods for detection of disease in calves. In this case, only naturally-occurring cases of disease will be assessed (disease will not be induced).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are assessing the welfare of adult cows and calves, so these are the most appropriate animals to use for these studies. No animal or computer models currently exist, but we will investigate these options if they arise from other work.

We have considered all possible alternatives to the proposed methods, and have concluded that the methods proposed are those that cause the least possible pain, suffering, distress or lasting harm to the animals whilst still giving meaningful results. Having meaningful and reliable results means that the experiment does not have to be repeated.

To understand the preferences of adult cows for lying areas, each cow must be able to express her choice without interference from other animals. This requires that each cow must be housed in a pen on her own to allow her to express her preference for lying areas. Lying is a behaviour that occurs at night, so several nights of data must be captured to give meaningful results.

The fixing of an eartag to calves is necessary because we are testing the robustness and validity of ear- mounted sensors to detect disease. Eartags are used for this type of monitoring as, once the ear has healed, they are less likely to get tangled in equipment compared to collar mounted sensors (which can result in injury) and to be outgrown (which can cause leg lesions) compared to leg-mounted sensors.
The blood sampling of calves is necessary, as we are trying to determine whether sampling saliva is a viable alternative to the analysis of blood in assessing dehydration. Measurements on blood are the gold standard against which we will compare the values found in saliva.

Taking a rectal temperature is necessary as it is the most accurate way of assessing an animal's temperature. The use of an indwelling temperature monitor has been considered, but cannot be used in this case as the animals eventually enter the food chain. Surgery would be required to implant and remove the indwelling sensor, or the animal euthanized early in life. Calves rapidly habituate to the handling required for assessing rectal

temperature.

Why can’t you use animals that are less sentient?

The issues that we are investigating are problems experienced directly by cattle and calves, so the use of these animals at this stage of life is the most direct way of solving the problems. There are no viable alternatives to the use of these life stages or any less sentient species that will respond in similar ways. We are assessing behavioural responses in some studies, which means that the use of terminally anaesthetised animals is not appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Increased monitoring of animals receiving procedures is the main way of minimising welfare harms. Cows housed in single-occupancy pens will be closely monitored for signs of distress. The ears of calves will be closely monitored for a number of days after insertion of the eartags to allow detection of any bruising or inflammation. After blood-sampling has taken place in calves, the wound site will be monitored for bleeding, and the behaviour will be monitored to ensure that normal behaviour such as feeding and resting is not disrupted.

Using staff who are well trained in the procedures is also important. Staff who have been trained to carry out the procedures will do them competently, quickly and effectively, thus minimising harm to the animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will keep up to date with updates to Home Office requirements and to 3Rs advice on their websites. New developments in the field of cattle welfare can be gathered from scientific publications and conference abstracts.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Information on advances in the 3Rs in this field are communicated through our institute's AWERB. Information on advances in methodologies are often communicated through scientific publications and at conferences, and we will stay informed about new developments via these channels.

87. Circadian rhythms in health and disease of the musculoskeletal 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Osteoarthritis, body clock, exercise, therapy, ageing

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how daily rest and activity patterns regulate skeletal tissue functions and protect us from age-related diseases. We also aim to investigate whether we can utilize our intrinsic body clock mechanisms to enhance tissue repair and improve treatment options for skeletal conditions, such as osteoarthritis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Osteoarthritis is common and debilitating, affecting 60% of people over the age of 65, with currently no cure and no disease modifying drugs. Numbers of people suffering from OA

will rise with increased longevity in the population and the disease represents a significant economic burden on the health service and is a major cause of decreased quality of life amongst the ageing population. There are currently no effective treatments for OA and joint replacement and analgesia are the only options for suffers of OA. Therefore, new understanding of how cartilage tissue is maintained on a daily basis and how this process goes wrong with ageing is urgently needed.

Body clocks are 24 hourly (circadian) rhythms driving key aspects of our physiology and behaviour, including the sleep/wake cycle. Recent research has demonstrated an essential role of the intrinsic clock timing mechanisms for normal function and structural integrity of the articular cartilage and other connective tissues. The program of work proposed here will help us understand how connective tissues such as those in the articular cartilage in our joints are maintained by our body clocks and daily rest/activity cycles, and why loss of this temporal control mechanism contributes to the development of osteoarthritis and other age-related diseases. The new knowledge gained in this project will help us understand why we develop this disease when we get older, and how we may be able to utilise the body clock mechanism to optimize treatment timings or design novel therapeutic approaches.

What outputs do you think you will see at the end of this project?

The research outputs of this project will include:

In depth understanding of how connective tissues in our skeletal system are formed and maintained on a daily basis, despite the routines of wear and tear associated with loading and activity. It is currently unknown whether and how exercise can reset our body clocks in the skeletal system. Proving our hypothesis in vivo in mice will allow us to identify a novel disease modifying intervention (timed daily exercise) for age-related skeletal diseases, such as osteoarthritis. These non-invasive approaches are likely to bring significant clinical benefits to patients experiencing osteoarthritic pain. This information will open new research avenues in the field of connective tissue research, arthritis, exercise biology and sports medicine.

A mouse model of osteoarthritis (OA) where clock genes of interest are selectively deleted in the cartilage of adult mice.

Data from our mouse model showing how body clocks influence the onset and progression of OA, both spontaneously and following surgical destabilisation of the knee joint. These will include imaging data and joint 'damage scores' to demonstrate whether OA develops more quickly and with greater severity in our model compared to controls, data on joint mobility and data on the levels of molecules that are associated with pathways that promote or inhibit the onset and development of OA.

Insights into the mechanisms by which body clocks have protective effects in joint tissues and how our drug candidates harness these mechanisms in the treatment of OA.

We will publish our findings in open access peer-reviewed journals making them available to the OA research community.

We will present our findings in the form of posters and oral presentations at conferences. The project will support our development of a disease-modifying treatment for OA.

Who or what will benefit from these outputs, and how?

These outputs have the potential to benefit patients with OA - a very common and debilitating joint disease that affects millions of people worldwide - by improving our understanding of the disease process and, ultimately, by supporting the development of new disease modifying treatment and interventions.

The outputs will also be of value to scientists who are conducting basic and clinical research in OA by providing new insights into the regulation of disease-associated mechanisms. This research will also shed new light on exercise biology and sports medicine.

We will also engage with media outlets (including newspapers, radio and television networks) to publicise our major findings and maximize impact of this work.

How will you look to maximise the outputs of this work?

We will maximise the outputs by disseminating the outcomes of our work through conferences, seminars and publications (of both successful and unsuccessful approaches). We will also engage with OA patients and their clinicians to ensure that our work to develop disease-modifying drugs or interventions (e.g. timed exercise) for OA is aligned with their priorities and needs. We will also collaborate with other researchers by making our knock-out mouse models available to other researchers.

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.

This project will utilise mice as a model of human osteoarthritis. We will also conduct treadmill exercises using young adult mice to investigate effects of exercise training on their skeletal circadian rhythms.

Use of a mouse model will allow us to carry out genetic modification to generate cartilage-specific gene knockouts in order to specifically explore our protein of interest in the knee joint during the development of OA. The generation of the gene knockout will require a breeding programme, which will use pregnant mice, embryos and all life stages from birth to adult mice.

Cartilage-specific gene knockout will be stimulated in young adult mice at the point when they have a mature musculoskeletal system. We will use these knockout mice to explore the spontaneous development of OA over a timeframe of up to 18 months.

We will also use the DMM (destabilisation of the medial meniscus) model of knee OA, where surgical destabilisation of the knee joint causes OA to develop over a few months. This model is used by many researchers in the OA field and is well accepted as providing

informative data on disease-associated pathways and the effects of therapeutic interventions.

Typically, what will be done to an animal used in your project?

The generation of genetically modified mice will involve natural mating of animals. The mice will be kept in standard conditions for up to 15 months. Some mice will be kept for up to 18 months without joint destabilisation surgery and then humanely killed so that joint tissues can be examined for molecular and cellular changes.

Mice with their clock molecules genetically engineered to carry either a light-emitting molecule or a deletion will undergo graded endurance training using a multi-user motorised treadmill, until running continuously for up to 60-mins per day, up to 5 days a week for up to 4 weeks. Each mouse will only go through one regimen of exercise. Animals will be acclimatised to both the environmental conditions (such as the mechanical noise of the motor) and to treadmill running which will gradually increase to the designed duration and speed. On completion of the exercise regime, mice will be killed for tissue collection and downstream analysis of circadian rhythms.

To induce cartilage-specific gene knockout, mice will be treated with tamoxifen. This will be administered either orally or by injection. To induce OA, mice will undergo a precise surgical procedure (under anaesthetic) to destabilise the knee joint. Animals will be humanely killed at a series of endpoints after surgery (up to a maximum of 20-weeks) so that joint tissues can be examined for molecular and cellular changes.

Some mice will be treated by a series of injections (up to a maximum of 1x/week, max 12 injections) with our drug candidates to improve disease outcomes.

As well as the endpoint examinations of knee joint tissues, the development of OA might also be examined using non-invasive imaging methods, such as x-ray and MRI (no more than once per month, max. 12 months), and by observation of the animals' mobility (a maximum of once per week).

Control mice, e.g. without gene knockout, will be included for comparison in all elements of the project.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected as a result of the breeding of genetically modified mice.

Adverse reactions are not anticipated for the treadmill running regimes we plan to use. Habituation to treadmill training may induce mild physiological and psychological stress. If an animal refuses to run during the habituation steps, it will be removed from the study. Short-term treatment with Tamoxifen can result in some weight loss; these effects will be short-lived.

Following joint destabilisation surgery, mice might experience some inflammation, discomfort and ultimately arthritis in their joint - i.e. a swollen joint and possibly limping. Our previous work in the DMM model has shown that, although we can see changes in the joint tissues at the endpoints of experiments, mice show very little sign of discomfort.

Injection with our drug candidates is not expected to have any adverse effects, based on our previous work.

Expected severity categories and the 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 breeding programme is a mild procedure. No animals are expected to experience adverse effects. The treadmill exercise is a mild procedure. No animals are expected to experience adverse effects.
The effects of tamoxifen treatment may be mild or moderate; this will be minimised by optimising the dose and route of delivery. Most animals will experience some effect.

The effects of DMM surgery is moderate. All or most animals will be affected.

The spontaneous development of OA in our gene deletion mice may be mild or moderate. We do not yet know the extent to which OA will develop in these mice.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are studying the complex biological process by which the articular cartilage is maintained through daily wear and tear, and subsequently can become degraded in osteoarthritis. The onset and progression of OA in humans is very variable. We consider that this is due to differences in intrinsic protective pathways within the joint. We have identified circadian timing mechanism in the cartilage as a critical regulator to inhibit OA-associated processes.

We are now at the stage of directly determining whether circadian rhythm disruption are pathogenic factors in the aetiology of OA and for these studies, there is no alternative but the in vivo model. Defining the molecular mechanisms involved and how they influence joint structure and function will improve our understanding of OA and support our development of new disease-modifying drugs. We make extensive use of cell culture models to study individual aspects of the process, but the holistic and complex process of connective tissue functions, including the aspects of the complex series of events leading to cartilage degradation associated with osteoarthritis cannot be studied in vitro.

The use of a mouse model is essential to achieve our aim. Mice have a musculoskeletal system that is similar to humans and are well established as informative models of OA. Mice are also the ideal species for genetic modification. The generation of a cartilage-specific gene knockout for our proteins of interest in young adult mice will allow us to specifically define the effects of clock genes within the knee joint. Previous studies have

already shown protective effects of circadian rhythms in mice and we are now uniquely placed to conduct the in vivo studies necessary to prove the causal link between chondrocyte circadian disruption and OA disease phenotypes and unravel the underlying mechanisms.

Mice mature much more rapidly than humans, so we will be able to look at the age-associated development of OA in our model over a relatively short timeframe (up to 18 months). In addition, surgical destabilisation of the knee joint in mice causes rapid onset of OA and is a very well accepted model of post-traumatic OA in humans. In these mouse models we will be able to use non-invasive methods to assess changes in joint mobility and structure during the study timeframes. At the study endpoints we will also be able to quantify joint damage and determine the expression of genes and proteins implicated in OA. In the joint destabilisation model we will be able to gather data for very early time points (from 6 hours after surgery) through to a maximum of 20 weeks. Evaluation of precisely defined time points, from the point of onset and throughout the progression of OA, could not be achieved using human tissues.

Before in vivo studies, we will test potential therapies in cell culture models to check that they have the expected effects in improving circadian rhythms and modulating the expression and activity of the clock related chondro-protective pathways.

Which non-animal alternatives did you consider for use in this project?

We have considered using clinical knee tissue samples from people with late-stage, symptomatic OA that are collected during joint replacement surgery, or early stage OA. We have already used these to explore how our protein of interest influences the expression of other proteins known to be associated with cartilage damage and how our drug candidate acts to suppress cartilage breakdown. We have also considered the use of cell culture models of cartilage, which we have used previously to generate mechanistic data.

Why were they not suitable?

The proposed work requires a model system that provides an accurate representation of the whole knee joint and where we can carry out analyses at precisely defined time points that represent OA onset through to late-stage disease. Human clinical samples are not suitable because they would not allow us to carry out genetic manipulation. Furthermore, there are no good diagnostic markers for early OA, and patients are seen by clinical teams at variable times following joint injuries, so it is impossible to identify patients at specific stages of the disease. Cell culture models could provide the option of genetic manipulation to knockout the protein encoding our gene of interest. However, OA is a disease of the whole joint and disease progression depends on the mechanical loading of the joint, which can only be achieved in a live 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 have estimated that up to 5000 mice will be involved in our breeding programme that will generate the genetically modified mice to be used for clock gene reporters and cartilage-specific knockout of our genes of interest. This is based on: (1) the number of experiments we plan to use these mice for, and
(2) the conservative assumption that each litter of mice we breed will include one mouse with the correct genotype.

We will use up to 500 mice for our studies on treadmill exercise. This is based on various timing and frequency of exercise protocols.

We will use up to 1000 mice for our studies on the spontaneous development of OA. In addition, we estimate that up to 400 mice will be used in the DMM model of OA. This is based on: (1) the different experimental time points we will use (in order to explore the effects of our protein of interest throughout the early stages of OA development), and (2) previous work by ourselves and collaborators using the DMM model, which suggests that each experimental group will need to include 10-12 mice in order to generate informative data. Among these mice for spontaneous or induced OA studies, we have estimated that up to 200 will be treated with Tamoxifen to stimulate cartilage-specific gene knockout. This is based on genetic targeting of 2 different clock genes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the outcomes of work in our own and collaborators labs and taken the advice of a statistician to reduce the number of mice being used in each experimental group. We will continually review the outcomes of experiments and use our data to optimise animal numbers as the project progresses.

By using both male and female mice in the spontaneous OA and DMM-induced OA studies we will reduce the numbers of genetically modified mice that we need to breed. Furthermore, generating data in both male and female animals is important with regard to the translation of our findings towards human clinical studies, where OA is common in both women and men.

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 continually review the outcomes of experiments and use our data to optimise animal numbers as the project progresses. We will also draw on our established local expertise for tamoxifen induction to ensure minimal animals are needed for the generation of cartilage-specific gene knockout.

We will follow the PREPARE guidelines (Smith et al 2017) to ensure the quality, reproducibility and translatability of our animal experiments.

To ensure efficient breeding, we will minimise the number of animals kept "on the shelf" and will breed, as far as possible, on demand. We will also have regular reviews of the colony performance. We have already optimised genotyping protocols.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use genetically modified mouse models to allow selective deletion of clock genes in the cartilage of young adult mice. This model will be used to explore the effects of the gene deletion on the onset and progression of knee osteoarthritis (OA). We will determine whether OA develops spontaneously in the knockout mice over time (up to 18 months); in this case we anticipate that any OA-like symptoms will be mild and slowly progressing. In genetically engineered mouse lines where there is an absence of any spontaneous OA phenotypes, we will initiate OA by surgical destabilisation of the knee joint in the DMM (destabilisation of the medial meniscus) mouse model. This is a very well characterised model, which we have used previously. We have observed that whilst mice develop OA- like damage to their cartilage, they exhibit little or no evidence of discomfort/pain over the timeframe of our proposed experiments.

Healthy mice enjoy running and it is an enriched experience for them to do so. Healthy wild type mice under standard animal house conditions will ‘run’ for 8-15 km per day in a running wheel. Indeed, we will use wheel running to confirm intact behavioural circadian rhythms in young adult mice with cartilage tissue-specific deletion of clock genes. However, voluntary running (e.g. through running wheel) is not suitable training apparatus for the specific aim in this project as previous work has shown that running wheels produce a heterogeneous response in their level of running. In addition, mice will not normally run at daytime. As such, treadmill is the methodology of choice because it is the only regimen that simultaneously allows for precise control of time of exercise, its intensity and volume.

Why can’t you use animals that are less sentient?

OA is a complex disease that involves the whole joint and is influenced by factors including joint mechanics as well as cellular and molecular processes. In order to explore the onset and development of human OA we need to use a live animal model with a mature musculoskeletal system that is similar to an adult human. The adult mouse fulfils this requirement. In addition, there are well-established methods for genetic modification and for surgical induction of OA in mice - both of which are essential for the proposed project. For the treadmill exercise study, again we need adult mice that have a mature musculoskeletal system that is similar to an adult human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Potential refinements include habituation for treadmill, increased monitoring, post-operative care and pain management.

The major welfare cost to the clock gene knockout mice in this study is the development of OA-like symptoms. Since the purpose of this project is to study OA in the mouse knee

joint, this welfare cost cannot be changed. However, any apparent pain associated with the onset of OA will be controlled by the use of appropriate analgesics as advised by the NVS with animals monitored to ensure adequate dosing.

With regards to the Tamoxifen induction of cartilage-specific gene knockout in our mouse model, we will continue to optimise the least harmful way of administering Tamoxifen in terms of the route, dosage, duration and intervals, whilst still ensuring effective knockout (determined by genotyping). Tamoxifen treatment can cause some weight loss but these are typically transient.

With regards to the DMM surgical model of OA, we will continually monitor our procedures for pre- and post-operative care to ensure that these are optimised. For example, using sterile techniques to prevent infection at surgical sites and making adjustments to food, bedding and enrichment and using postoperative analgesics if required. In addition, we will carry out pilot studies and order our experiments so that the data we gather can be used to refine the numbers of animals being used at each stage, i.e. to ensure that all experiments are informative, but without using more animals than necessary. As well as collecting data on joint damage and gene expression profiles at the end of each experiment, we will use non-invasive methods (such as imaging and observation of animals' mobility) during the course of each experiment so as to maximise the information we obtain, but without any additional welfare costs to the mice.

With regards to the treadmill exercise experiments, experience from a local colleague shows that adverse reactions are not anticipated for the running regimes we plan to use. Habituation to treadmill training may induce mild physiological and psychological stress. To mitigate this, animals will be acclimatised to both the environmental conditions and to treadmill running. Animals will be monitored for adverse signs such as fatigue and indications of injury, which if they occur the exercise will be halted.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use published documents recommended by NC3Rs to ensure that we are using the most refined approaches in all our experiments.

We will closely follow the best and refined practice on GA breeding, Aseptic surgery, Prepare guidelines, Pilot studies, Systematic review, Enrichment, Social housing, Tube handling and 3Rs assessment, as listed on the NC3Rs website.

With regards to DMM model of OA, we will regularly review the scientific literature for work by other researchers in the field to identify opportunities for refinement. In terms of treadmill exercise, we will closely review the scientific literature for similar work from other groups to refine our protocols.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are registered with NC3Rs and subscribe to their monthly newsletters, which will keep us informed regarding advances in the 3Rs. We also have access to an NC3Rs Regional Programme Manager. We will utilise the NC3Rs Experimental Design Assistant as well as seeking guidance from our local NVS and NACWO to inform the implementation of any advances that can be applied to our project.

88. Efficacy of anticoccidials and antiprotozoals against protozoal diseases in farm animals and farmed birds.

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

Coccidia, Eimeria, Cryptosporidium, Anticoccidials, Antiprotozoals

Animal types	Life stages
Cattle	juvenile, adult, pregnant, neonate
Sheep	adult, juvenile, neonate, pregnant
Goats	adult, juvenile, neonate, pregnant
Pigs	juvenile, adult, pregnant, neonate
Domestic fowl (Gallus gallus domesticus)	juvenile, adult, neonate
Domestic poultry	juvenile, adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To propagate protozoa, particularly coccidian and to test anticoccidials and antiprotozoals for efficacy against coccidiosis in the target species. The target species will be infected either naturally or artificially then treated (may treat or commence treatment prior to infection). Following on from this we will examine parasite excretion levels in faeces and e.g. perform post-mortem assessment of intestinal lesions in order to confirm the efficacy for a selected anticoccidial and/or antiprotozoal.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 assess the efficacy of novel and varying dose rates of anticoccidial and antiprotozoal therapeutic agents on coccidiosis in a range of target animals. Coccidiosis is a ubiquitous disease and results in the loss of €2.3billion worldwide in poultry (mainly Eimeria) and $120million worldwide in other livestock species (Eimeria and Cryptosporidium). There are also many other important protozoal diseases of livestock that cause economic losses. These include histomoniasis, Spironucleus and Trichomonas. This work will help to contribute towards better understanding and control of animal diseases and may lead to novel veterinary medicines reaching the market to combat coccidiosis and other protozoal disease of animals (such as cryptosporidiosis, Babesia and giardiosis).

What outputs do you think you will see at the end of this project?

Contribution to the development of new anticoccidial and other antiprotozoal treatments with the possibility of scientific papers being produced. The result of the projects will be scientific reports that will help progress the understanding and treatment of protozoal infections within farm animals, with many contributing to regulatory submissions.

Who or what will benefit from these outputs, and how?

The direct benefits will be a series of study reports, normally one for each of the studies conducted. These provide the information that the client needs to support, for example, their claims and/or applications for veterinary medicine or food additive claims.

Indirect benefits will be the contribution towards helping to control protozoal diseases in animals which is a significant cause of suffering to animals and economic cost to the community. There is a constant need to combat the development of drug resistance by parasites, which may be brought about by adapting management techniques and existing products or by novel approaches. Compounds that act in novel ways have to be developed, tested for efficacy and brought to market. Existing products need to be evaluated against current field strains to ensure that they remain effective.

Financial income for the company enabling further contract research studies.

How will you look to maximise the outputs of this work?

We will work with global companies, institutes and charities to disseminate the study findings, publish research papers, attend relevant conferences and we also play an active role in creating guidelines in this area of research.

Species and numbers of animals expected to be used

- Cattle: 300
- Sheep: 200
- Goats: 50
- Pigs: 150
- Domestic fowl (Gallus gallus domesticus): 4000
- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Many of our studies are conducted in the target species for authorisation or support of, for example, a veterinary medicine and such testing is required by the regulatory authorities. There is a policy of checking whether non-animal alternatives are possible, for example by regular visits to the NC3Rs website (http://www.nc3rs.org.uk) and attending relevant conferences. Where there is a validated in vitro model then the client would be informed and the in-vivo work refused.

Typically, what will be done to an animal used in your project?

They may be infected either for passaging, dose determination purposes or for artificial infection prior to or following or during the administration of a treatment. Artificial infection may be done via oral gavage, exposure in the environment or insertion in the cloaca.

Treatment may be administered orally in feed, water or using a gavage. Subcutaneous, intramuscular or intravenous injection may be used, also a subcutaneous slow release device may be used. The treatment may also be applied topically to the skin in the form of a spray or pour-on.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals are expected to tolerate the administration procedures with a maximum of transient discomfort.

Often there is a requirement to use a recently isolated field strain whose pathogenicity is characterised during the initial passage(s).

There is an expectation that clinical disease will develop and bloody/mucoid diarrhoea is expected in infected individuals.

Where species are mixed there is an attempt to get the combination and levels in the strain into proportions to show the required production and pathology impact without unnecessary clinical disease.

In order to mitigate the risk of clinical disease (diarrhoea, significant morbidity and mortality in some cases), the strain to be used in a study is normally tested in a small pilot first.

Extra checks are put in place throughout each 24 hour period at key times after infection (in both pilots and main studies) to coincide with expected timing of clinical signs in order to make sure that animals can be euthanased if necessary before exceeding the moderate severity limit. The intervals between checks are determined on a case by case basis and are normally at a minimum of one hour.

Typically animals infected with coccidia show a peak of clinical signs for 3 – 4 days in each cycle. Thereafter subsequent cycles (in studies such as floor pen studies that

continue past the first infection cycle) are normally less severe as the animals are older and more immune.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

25% mild and 75% moderate severity (see full breakdown in protocols). The maximum expected severity is moderate with any animals that reach this threshold being treated or euthanased as appropriate before they reach the severe threshold.

Animal types	Est. numbers (mild)	Est. numbers (moderate)	Life stages
Cattle	75	225	Neonate, Juvenile,Adult, Pregnant
Sheep	50	150	Neonate, Juvenile,Adult, Pregnant
Goats	12	38	Neonate, Juvenile,Adult, Pregnant
Pigs	37	113	Neonate, Juvenile,Adult, Pregnant
Birds	2000	6000	Neonate, Juvenile, Adult

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Anticoccidial and antiprotozoal veterinary medicines, biologicals, feeds and feed additives must be trialled in the target species for initial safety/tolerance and efficacy before taking forwards to regulatory submission or the market. Unless there is a viable alternative such as an established and validated tissue model available, the target animal will need to be used in these studies.

Which non-animal alternatives did you consider for use in this project?
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?

We use a combination of tools to estimate the numbers of animals needed. This normally includes the NC3Rs Experimental Design Assistant, VICH and WAAVP guidelines as well as consulting guidelines set by regulatory bodies and any relevant literature to ensure that the minimum number of animals is used.

Peer-reviewed journals and advice from external peers together with in-house experience and historical data over a number of years is used to ensure that animal numbers are adequate.

We are fully committed to reduction, nonetheless the exact numbers of animals required will vary with the particular project needs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have many years of experience of working with coccidial and protozoal infections, passaging and treatment regimes. This ensures the minimum number of animals are being used for any particular study. Due to our experience we have optimised our processes which enables us to maintain high welfare standards whilst delivering studies that meet requirements. We utilise resources such as the NC3Rs Experimental Design Assistant, VICH and WAAVP guidelines as well as consulting guidelines set by regulatory bodies and other relevant literature.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies are used to establish an appropriate infection level prior to the start of a main study. This ensures a consistent and safe infection level resulting in meaningful and valid results without compromising on animal welfare and safety.
Wherever possible the pilot study will be conducted in the same proposed breed or strain of animal as the main study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm

to the animals.

The animals used are the target species for the veterinary medicines or other product being studied. Any infection models will have welfare at the centre of their design.

In addition, animals on these studies will have a heightened level of observations immediately after and, where necessary, in the days and nights following administration of any substance, particularly around anticipated peaks of clinical signs associated with a particular infection.

If clinical signs are approaching or have breached the severity limits in place, then the Establishment Licence Holder, Project Licence Holder, NACWO(s), NVS and, where appropriate, a member of the Animals in Science Regulation Inspection Unit will be consulted to decide the course of action. This could involve immediate alleviating of suffering via euthanasia, or treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering.

Why can't you use animals that are less sentient?

We have to use these animals as hosts due to the research being directly related to treatment and/or prevention of infection in these species.

However we regularly refine existing and/or validate animal models consistent with 3Rs initiatives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?
We minimise suffering by examining each study plan to identify opportunities to reduce handling by, for example, using experienced and knowledgeable staff, habituating animals to procedures where necessary, meeting or exceeding codes of practice and current guidance or literature suggestions for enrichment.

The animals used are the target species for the anticoccidial or antiprotozoal product on trial. Any models will have welfare at the centre of their design. In addition, animals on these studies will have a heightened level of observations immediately after and in the days following administration of any substance. RRL's standard level of monitoring is a minimum of twice daily. Frequency of observations are increased on an as needed basis based on animal observations and behaviour. For example, observation frequency would increase to hourly, overnight monitoring following the appearance of clinical signs associated with coccidia infection. Observations would normally be general health observations but these are supplemented with clinical observations by our NVS (or similar), monitoring oftemperature and other parameters as necessary.

If a treatment is to be administered over the course of a number of days, such as in feed or water, then we would aim to administer at the same time daily to allow the animal to become accustomed to the dosing regimen. We may also associate dosing and administration with feeding times as positive reinforcement for the animal

If it is believed an animal is approaching and likely to breach the predetermined severity category then we have a well-established process. This includes seeking veterinary advice from our NVS or another veterinary surgeon, discussions with the NACWOs, PELh, PPLh, PILh and Study Investigator. This group of people decide the best next steps in terms of animal welfare. This could be either, immediate alleviating of suffering if irreversible clinical

signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, and increased monitoring if the animal is unlikely to breach the severity limit by the conclusion of the study if appropriate.

If an animal breaches the predetermined severity category for the study then immediate action is taken. This often includes rapid discussions with the NVS, PELh, PPLh and PILh alongside the study investigator. We would also, if possible, contact an ASRU inspector. The course of action could be immediate alleviating of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, maintaining and closely monitoring the animal if the scientific need is justified and the end of the study is within a short time frame, if agreed by an ASRU inspector. All decisions are taken on a study by study basis and will differ with treatments and species.

We will also continually review our procedures and refine as much as possible with guidance from, for example, VICH, WAAVP, EMA, and other relevant organisations and published literature.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

World Association for the Advancement of Veterinary Parasitology (WAAVP) and VICH guidelines for best practice. These guidelines are now seen by most regulatory authorities as the standard by which parasite-related protocols are written, and consider numbers of parasites required for study purposes.

Suitable literature will be used to stay up to date with husbandry practices to ensure that initiatives for e.g. enrichment can be assessed and incorporated as appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly reference the NC3Rs website, published literature, attend relevant conferences and employees are members of The Royal Society of Biology and other relevant organisations.

89. Amphibian egg extracts as tools for understanding genome stability

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, cell division, genome stability, mutations, DNA replication

Animal types	Life stages
Xenopus laevis	adult, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Genetic instability is a characteristic of a large proportion of cancers and occurs most often when the DNA in a cell is being copied (replicated), before the cell divides. The aim of the research programme is to understand the pathways used by normal cells to prevent defects in the copying process, or failing that to induce cell death without further division.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 of the basic biology that underpins genetic stability is important for the development of drugs to treat diseases such as cancer where this becomes defective. Several of the pathways that the lab has played a role in elucidating have been targeted by anti-cancer drugs that are being trialled in the clinic.

What outputs do you think you will see at the end of this project?

We will have a better understanding at the molecular and biochemical level of the pathways used by cells to prevent genetic instability and will have a better understanding of what happens when these pathways are blocked or inhibited. This in turn may lead to new ideas about how genetic instability could be targeted in diseases such as cancer. The results will be presented in a number of publications.

Who or what will benefit from these outputs, and how?

Beneficiaries will be other scientists working in related disciplines and biotechnology and pharmaceutical companies interested in developing new therapies for diseases such as cancer.

How will you look to maximise the outputs of this work?

We will continue collaborations with scientists around the world as appropriate. Where new compounds with potential clinical use become available we will examine their effect on pathways relevant to our work and will make the results freely available. If we discover new potential drug targets we will engage with other researchers or biotechnology and pharmaceutical companies to explore the feasibility of developing a drug discovery campaign.

Species and numbers of animals expected to be used

- Xenopus laevis: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cell-free extracts of Xenopus eggs provide the only currently available system for supporting cell cycle progress in vitro. They therefore offer a unique opportunity to investigate genome stability pathways at a biochemical level.

Typically, what will be done to an animal used in your project?

Females will be injected with a hormone that naturally induces egg laying; they then lay eggs over the next day. Females are then rested for 4 months before being used again for egg-laying.

What are the expected impacts and/or adverse effects for the animals during your project?

Frogs experience transient discomfort as they are being injected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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-free extracts of Xenopus eggs provide the only currently available system for supporting cell cycle progress in vitro. They therefore offer a unique opportunity to investigate genome stability pathways at a biochemical level.

Which non-animal alternatives did you consider for use in this project?

The lab uses and continues to use a number of non-animal alternatives, including mammalian tissue culture cells and recombinant proteins.

Why were they not suitable?

Whilst mammalian tissue culture cells and recombinant proteins are very useful and have provided much valuable information, they ultimately cannot substitute for the unique combination of attributes provided by egg extract: a physiological system where genome stability pathways can be analysed and manipulated biochemically.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 know approximately the volume of egg extract that we will require each year to perform our planned experiments. From our knowledge of the average amount of useable extract obtained from each female, we calculate the total number ofanimals required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have reduced the number of animals required in several ways: i) freezing extract so that none goes to waste; ii) minimising the amount of extract required in each experiment; iii) maximising the number of eggs produced by each female; and iv) improving the quality and quantity of extract by continual refinements to the extract-making protocol.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As soon as possible during the experimental phases of the project we will move our discoveries from the Xenopus system to other non-animal systems such as mammalian tissue culture cells and recombinant proteins, either by undertaking the work ourselves or by collaborations with experts in the relevant systems.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use cell-free extracts of Xenopus eggs which faithfully recreate the nuclear events of the cell division cycle in vitro. In order to induce egg-laying, captive frogs must be injected with hormones, which causes only transient discomfort. We have investigated in some detail the possibility of adding hormones to the water the frogs live in, but this yields small and unreproducible numbers of laid eggs; despite trying a large range of different conditions for doing this, we have not been able to significantly increase yield or reproducibility using this method.

Why can’t you use animals that are less sentient?

Adult females are required to lay eggs. No comparable system has been developed from less sentient animals (a comparable extract from Drosophila eggs has a large number of technical drawbacks compared to the Xenopus system).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will ensure that the housing and husbandry is of the highest quality, as the health and wellbeing of the frogs has a very large influence on the quality of eggs produced. We will continue to improve extract procedures to maximise the usefulness of the extracts and to minimise the number of unusable low- quality eggs that are laid.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the most up-to-date protocols published in the scientific literature relating to animal husbandry, egg production and extract preparation.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to communicate with other Xenopus researchers to learn of new technical developments and refinements in the field.

90. Diet Induced Obesity model 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

Key words

Obesity, Metabolic, Diabetes, Cholesterol

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This is a licence in which the programme of work is designed to produce prepared rodents and/or use of rodents to conduct basic early stage research. The rodents form the basis of several different animal models of diseases such as such as type 2 diabetes, high blood pressure, high cholesterol and other diet induced circulatory problems. Essentially animals are fed a high fat diet or high fat/high sugar diet for a number of weeks. As a result, they become obese, and usually have high blood sugar, and develop impaired sugar tolerance. These animals are then used to study the adverse clinical and genetic effects of obesity and type 2 diabetes and to test drug candidates that are expected to affect these conditions in humans. This is a demand-led service supported by a scientifically justified need. We will never exceed the moderate severity limit. The scientific background for each individual use is specific to each researcher but broadly addresses scientific and clinical needs in the following areas of research: obesity and metabolic related 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 benefits of the service program are therefore to provide facility for the scientific community to complete these programs of work where resources may otherwise have been limited. The availability of appropriate facility and expertise then ensures the procedures will be performed to a consistently high standard allowing greater reproducibility and ensuring the highest standards of rodent care.

What outputs do you think you will see at the end of this project?

As a result of conditioning rodents to become obese through the alteration of diet, clients can conduct basic early stage research. The rodents form the basis of several different animal models of diseases such as such as type 2 diabetes, high blood pressure, high cholesterol and other diet induced circulatory problems.

Who or what will benefit from these outputs, and how?

As this is a service licence the client will benefit upon receipt of the obese model as this will facilitate their area of research.

Therefore, this project will contribute to the achievement of scientific benefit on clients' own PPLs e.g. wider scientific community and patients.

How will you look to maximise the outputs of this work?

As this is a service licence, the work will be led by customer demand and will be managed centrally to ensure the 3R's are observed under this licence before shipping to the customer licence.

Species and numbers of animals expected to be used

- Mice: 7000
- Rats: 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.

Adult mice and rats are good models for basic research into obesity and develop similar obesity related complications to humans. Multiple studies have been conducted in rodents to demonstrate the translational effectiveness of these animals in biomedical research.

Typically, what will be done to an animal used in your project?

Essentially animals are fed a high fat diet or high fat/high sugar diet for a number of weeks. As a result, they become obese, and usually have high blood sugar, and develop impaired sugar tolerance. These animals are then used to study the adverse clinical and genetic effects of obesity and diseases such as type 2 diabetes and enable the researcher to test drug candidates that are expected to affect these conditions in humans.

During a typical procedure a small piece of ear tissue may be obtained to allow for genetic analysis, blood samples (No more than 10% of estimated circulating blood volume within a 24hr period or >15% of estimated circulating blood volume in 28days), administration of diets of altered composition and/or medicated feed and /or drinking water to modulate/induce obesity or other obesity related diseases (e.g. atherosclerosis) , and potentially administration of glucose or insulin via injection for glucose/insulin tolerance testing.

What are the expected impacts and/or adverse effects for the animals during your project?

Some animals will develop diabetes. These animals will be closely monitored for signs such as excessive urination or water consumption compared to control animals and appropriate husbandry procedures will be employed to ensure adequate dry bedding and water is available to minimise likelihood of welfare impact from cage environment.

Blood samples will be taken from superficial blood vessels with no adverse effect expected.

Injections carry a risk of local inflammatory lesions, but this will be reduced or eliminated through keeping the administration to the minimum volume compatible with the scientific objectives.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals will become obese and may develop moderate clinical signs associated with obesity, such as type 2 diabetes.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are essential for research into human and animal diseases, as behavioural models and full system responses cannot be replicated using non-animal methods. Our Establishment will make consideration to the use of alternatives for every study and if unable to replace the use of live animals, will identify the most appropriate reduction strategies for the research work.

Which non-animal alternatives did you consider for use in this project?

There are no alternatives to using live animals for this area of research.

Why were they not suitable?

Full system responses cannot be replicated using non-animal 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?

This licence is a service licence and therefore numbers estimated are driven from historical customer demand for the service.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Consideration to identifying the most appropriate ways to reduce the numbers of animals used for their research work will be discussed with every new client project enquiry and ask them to confirm that they have received statistical advice on their experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our AWERB will assess the project proposal internally for all new DIO clients/protocols. Recommendation will be made to reduce the number of animals used to the minimum required to safely deliver a valid result, use of pilot studies to define the optimum schedules for induction/treatment will be considered.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Wild type and genetically altered rats and mice will be used in this project.

This project is of a moderate severity and will involve feeding animals a higher fat/calorific diet to bring on the onset of obesity. There are no other less stressful methods to produce a reliable obese model mimicking human obesity related complications.

Why can’t you use animals that are less sentient?

A mammal is needed to compare to human anatomy therefore rodents are used as the least sentient mammal used in research

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All studies will have ethical and welfare review before starting and this will ensure minimal costs to the animal will occur. All clients will need to ensure statistical analysis has been completed to ensure the minimum number of animals are used in order to meet the scientific outcome.

For diabetic animals there will be increased monitoring of animals that develop polyuria and polydipsia
– twice weekly bedding changes and water bottle refilling, and the use of body condition scores will aid welfare assessment and humane endpoints.

We will minimise tail handling and encourage the use of non-tail handling techniques for routine husbandry procedures, such as cupping.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3R's Experimental design and reporting.

NC3R's Guidelines for the Design and Statistical Analysis of Experiments Using Laboratory Animals,
Michael F. W. Festing and Douglas G.Altman

The ARRIVE guidelines 2.0

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All studies will have ethical review and each client will be asked to justify the ethical use of animals. This project supplies animals that become obese for further research.

I attend industry conferences and ensure all learning on the 3R's are applied to my project where possible.

91. Microbial and immune drivers of infection in chronic lung disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Asthma, Chronic obstructive pulmonary disease, Immunology, Microbiome, Respiratory infection

Animal types	Life stages
Mice	adult, aged, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The Aims of this project are:

To understand the role played by the lung microbiome ('resident' bacteria that are present in the normal lungs) in controlling how the body's immune system responds to harmful viruses, bacteria or fungi.

To determine how changes that occur in the lung microbiome of patients with the conditions asthma and COPD (chronic obstructive pulmonary disease, a smoking related disorder) predisposes these individuals to infections by harmful viruses, bacteria or fungi.

To study whether we could administer specific bacteria normally present in the microbiome of healthy people into the airways of patients with asthma and COPD to alter their microbiome and boost their immune system's response to harmful viruses, bacteria or fungi.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic lung diseases including asthma and chronic obstructive pulmonary disease (COPD) are major causes of death worldwide. Patients with these conditions are prone to infections by harmful viruses, bacteria and fungi that lead to acute symptom flare-ups ('exacerbations'), devastating episodes with a 1 in 4 mortality rate. We urgently need to improve our understanding of why some patients are more susceptible to infections. We could use this understanding to develop new ways of treating the condition with the aim of improving quality of life and outcomes for patients.

Recent studies have revealed that the lungs are colonised by millions of resident 'commensal' bacteria ('microbiome') which may help to protect against infections triggered by other more harmful bacteria. Asthma and COPD patients show outgrowth of certain commensals within their microbiome. We do not know whether this outgrowth is just a consequence of the disease or if, by upsetting the immune system, specific commensals actually contribute to increased susceptibility to infection by harmful viruses, bacteria or fungi.

This projects asks the following questions:

Do commensals in the lung have a general protective role in preventing infections by harmful viruses, bacteria or fungi?

Do specific changes in the microbiome that occur in asthma or COPD reduce the immune system's ability to fight infection by harmful viruses, bacteria or fungi?

Could we administer commensals from healthy people into patients with asthma or COPD to improve their immune system's ability to fight off infection?

We will take a two-pronged approach to answer these questions. Firstly, we will use human samples to study if asthmatic or COPD patients with frequent severe infections have outgrowth of certain commensal bacteria within their microbiome. We will then conduct experiments in mouse models that are not ethically or technically possible in humans to deplete or manipulate the lung microbiome by introducing commensals that are found to be increased in patients with asthma and COPD. We will study how these commensals directly affect the immune system's ability to fight off harmful viruses, bacteria or fungi.

It is anticipated that this work will identify important commensals that can directly upset or enhance the immune system. This would then lead to development of new therapies where healthy commensals could be introduced into the lungs of patients to boost their immune response when exposed to harmful bacteria, viruses or fungi. In summary, this research will provide new scientific insight into an area of huge clinical importance and facilitate development of exciting new therapies to reduce the burden of disease caused by asthma and COPD.

What outputs do you think you will see at the end of this project?

Existing respiratory microbiome studies have generated lists of commensals present in asthma and COPD with no clear understanding about how these changes contribute to symptoms or susceptibility to harmful infections. The current proposal will address this major gap in our understanding by providing new insight into how the respiratory tract microbiome interacts with the immune system to control protection against infection and how these processes are disturbed in people with asthma or COPD. This will lead to a number of tangible outputs, as detailed below:

Fundamental new knowledge on the critical microbial regulators of host defense to respiratory infection and how these factors are altered in chronic lung disease. The role of the microbiome at other body sites (e.g. the gut) is well established but very little is known about if and how the lung microbiome regulates protection or susceptibility to harmful infections and the key members of the microbiome involved are unknown. This project will provide detailed insight into this area for the first time.

New therapies for treating patients. Through our fundamental work, we aim to identify important commensals that can directly upset or enhance the immune system. This would then lead to development of new therapies where healthy commensal bacteria or bacterial products could be introduced into the lungs of patients with asthma or COPD to boost their immune response when exposed to harmful viruses, bacteria or fungi.

Research Publications. New knowledge and techniques developed in this project will be published in peer-reviewed scientific journals with early deposition on 'preprint' servers (e.g. BioRxiV) to ensure rapid dissemination to the wider community.

Data sharing. Certain aspects of the work planned will include 'sequencing' technologies where large datasets of genes, proteins or commensal bacteria are generated from lung tissue samples collected within experiments. These data will be deposited on public repositories to allow other researchers to access the data and use it in their own studies, thereby maximising the potential wider benefits of the results generated.

Future potential to reduce animal usage. Many animal disease models show large degrees of variation when attempts are made to replicate them in different animal facilities. The reasons for this are unknown but one theory is that animals in different facilities may have variations in their microbiome. Through understanding how different commensal bacterial groups affect the immune system, we may be able to yield insight that will better control variation between facilities and therefore produce more consistent models to reduce animal usage long-term.

Who or what will benefit from these outputs, and how?

This project will provide a major scientific advance that will be of relevance to a range of disciplines within the UK and internationally. We envisage benefits arising from several areas that are all achievable within the period of this PPL and will also extend long-term.

Benefit to other respiratory researchers. There is growing interest in the airway microbiome in respiratory disease. Although our proposal focusses on the microbiome in the context of asthma and COPD, respiratory tract microbiome changes occur in many chronic lung conditions (e.g. cystic fibrosis, idiopathic pulmonary fibrosis etc.) and our findings will be of broad interest to researchers in these areas. Short term benefits (<5 years) include research publications, training of new staff, new research collaborations, conference presentations and development of new experimental protocols. Longer term benefits (>5 years) include establishing a framework for future studies where causality can

be examined using the models we develop, potential new Intellectual Property (IP) opportunities including new drug candidates and further grant funding based on successful track record.

Benefit to Immunologists: Outside of the respiratory field, this proposal will also examine fundamental questions regarding how commensal bacteria can shape the immune system and how disturbances in microbiota composition affect the immune system's response to infection generally. This insight will have wide implications for basic immunologists interested in this area. Short term benefits (<5 years) include research publications, training of new staff, new research collaborations, conference presentations and development of new experimental protocols. Longer term benefits (>5 years) include establishing a framework for future studies where causation can be examined using the models we develop, potential new IP opportunities including new drug opportunities and further grant funding based on successful track record.

Benefit to animal researchers and suppliers: Our research may provide crucial insight into why certain animal models cannot be reproduced by independent groups. In some cases, this could be dictated by differences in the respiratory microbiome composition of animals used to replicate studies (presence or absence of certain commensals). This will have broad benefit to researchers working in academic and industrial animal facilities and additionally for commercial animal suppliers (who may use the information to standardise the microbiome of their mouse lines). Short-term benefits (<5 years) include publications in journals specialising in laboratory animal science, presentations at conferences with a similar focus and refinement of existing models to better control for lung microbiome variability. Longer term benefits (> 5 years) relate to new insight that will allow researchers in the future to better control these variables and development of better commercially available mouse lines through standardisation of microbiota.

Benefit to patients: Asthma and COPD are major causes of mortality worldwide with very few effective therapies. Our research will gain better understanding of the mechanisms that underlie increased infection susceptibility in affected patients and drive development of new effective therapies for these devastating conditions. This work will not only identify new therapeutic targets but provide new models to demonstrate 'proof of concept' before therapies can be moved into clinical trials. Short- term benefits (<5 years) include better definition of patient sub-groups that gain benefit from existing therapies, better awareness of these conditions through public engagement of findings we generate and acceleration of the drug discovery pipeline. Long-term benefits include development of new 'probiotic' therapies or anti-bacterial/anti-viral/anti-fungal agents that can be incorporated into clinical practice.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through the following routes:
Public engagement: We will communicate the findings of this research to the general public in a format that is easily understandable to justify importance and disseminate key findings. This will occur through a range of platforms including social media, press releases, newsletters and participation in Science Festivals.

Presentation at conferences: We will disseminate our findings with other respiratory, immunology and animal model researchers through presentation of data at national and international conferences.

Scientific publications: We will publish our protocols and results within research papers in scientific journals (as open access manuscripts with early deposition on preprint servers) and through other platforms including online data repositories.

Communication with patient groups.We will liaise with relevant patient groups as a two-way process to discuss key findings generated during this project and use their input to guide future research directions and results dissemination. We have strong links to the British Lung Foundation and Asthma UK and will organise 'Meet the Research Team' sessions for patients and employees of these organisations to effectively disseminate our findings within UK and international patient communities.

Translation of findings into human experimental studies: As clinical academics we are well positioned to rapidly translate our mechanistic findings into clinical trials of promising therapies. We have a number of established clinical and industrial collaborations providing strong support to the feasibility of this goal.

Species and numbers of animals expected to be used

- Mice: 7600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 largely uses adult mice (since both asthma and COPD affect adult subjects) with some experiments using 'aged' mice (as COPD is a disease that frequently affects elderly individuals). The decision to use a mouse model was made after careful consideration based on the requirement that we seek to definitively understand complex mechanisms underlying how the microbiome acts to control the immune system. A mouse model was essential as it is not possible to recreate the complexities of lung bacterial communities in cells cultured in the laboratory and it is also currently not ethically or technically possible to deplete or manipulate the airway microbiome in human subjects. Using the mouse model therefore provides an experimental system where we can manipulate the human microbiome in a controlled way that is relevant to human disease.

Mice are closely related to humans in terms of similarity of genetics and immune system function so that experiments done can be directly related to human disease. Mouse models also have the advantage of having readily available reagents (antibodies) that can be used to block specific substances that are produced by the immune system in response to the microbiome. We also have a long track record of carrying out experiments in mice and we have already set up many of the models we plan to use in this project.

Typically, what will be done to an animal used in your project?

A typical experiment would involve mice receiving a light inhaled anaesthetic to allow intranasal (through the nose into the lungs) administration of antibiotics. This would occur daily for five days to deplete the existing lung microbiome. Using a similar method, we would then intranasally administer commensal bacteria (of the type seen in the lungs of asthmatic or COPD patients) for three days to mimic the microbiome of these conditions.

This would be followed by intranasal administration of a virus, bacteria or fungus that causes disease in humans (e.g rhinovirus, Streptococcus pneumoniae or Aspergillus Fumigatus). Animals are visually monitored every day during this experimental protocol and also weighed twice per week to ensure the infection is not causing weight loss which is a sign of ill health. Animals would typically be humanely killed at early (8, 24 hours after infection) and later timepoints (4 and 7 days after infection) and we would take a range of samples to study the immune response to the infection in detail.

In some experiments, we will administer substances into the lungs of mice to induce changes consistent with asthma. This would typically involve mice again receiving a light inhaled anaesthetic to allow intranasal administration of house dust mite (a substance that induces an allergic reaction in the lungs that mimics asthma). This would occur three times per week for a total of three weeks. Following this, mice will again receive intranasal administration of commensal bacteria for three days followed by administration of a virus, bacteria or fungus. Animals are visually monitored every day during this experimental protocol and also weighed twice per week to ensure the infection is not causing weight loss which is a sign of ill health. Animals would again be humanely killed at early (e.g. 24 hours) and late (e.g. 4 days) timepoints and we would take a range of samples to study the immune response.

In other experiments, we will similarly administer substances into the lungs of mice to induce changes consistent with COPD. This would typically involve mice receiving a light inhaled anaesthetic to allow intranasal administration of a single dose of elastase (an substance that induces lung changes similar to COPD). Ten days later (when COPD changes become established), mice will again receive intranasal administration of commensal bacteria for three days followed by administration of a virus, bacteria or fungus. Animals are visually monitored every day during this experimental protocol and also weighed twice per week to ensure the infection is not causing weight loss which is a sign of ill health. Animals would again be humanely killed at early (e.g. 24 hours) and late (e.g. 4 days) timepoints and we would take a range of samples to study the immune response.

In some of the above described experiments, we will also measure how these manipulations affect the 'function' of the lungs. This will typically (~90% of experiments where lung function is required) be done using a technique called 'whole body plethysmography' where mice are placed in small individual chambers for around 50 minutes which are connected to sensors that can measure the breathing rate and depth. We typically administer a aerosol 'bronchoconstrictor' (substance that briefly and mildly challenges the breathing) into the chamber to see how the breathing rate and depth responds. Mice are closely monitored for any signs of distress throughout this process.
In some experiments involving the above described models, we will also use mice that have been bred to specifically lack or over-express certain genes that encode immune substances in the lungs. By examining how this affects immune responses (in comparison to mice with normal levels of the gene), we can determine whether the gene plays an important role in dictating the immune response.

What are the expected impacts and/or adverse effects for the animals during your project?

Intranasal administration of viruses, bacteria or fungi into mice may induce varying degrees of 'inflammation' (caused by white blood cells flooding into the lungs to fight infection). This inflammation can lead to clinical signs. We are interested in evaluating this inflammatory response to understand how the presence of specific commensal bacteria

may affect this. Depending on the type of viral, bacterial or fungal infection and/or dose some animals will occasionally develop mild to moderate symptoms, consisting of reduced activity, hunched posture and tachypnoea (breathing rate faster than normally expected). These effects would generally commence around 48 hours after infection and may persist until the end of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 75% of mice are likely to experience moderate levels of severity. The remaining 25% of mice are likely to experience mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The decision to use a mouse model was made after careful consideration in comparison to other alternative approaches. The overall goal of this project is to definitively understand the mechanisms underlying how the lung microbiome interacts with the immune system to regulate protection against infections in health and promote susceptibility to infections in patients with disease (asthma or COPD). To do this, we needed an experimental system that: (i) allowed us to deplete or manipulate the airway microbiome to understand if it plays a causal role in the immune system's response and (ii) allowed us to block substances produced by the immune system that may be under control by the microbiome.

A mouse model is the only realistic option that would allow us to carry out these types of studies in a controlled manner that is still relevant to human disease and it is therefore essential that we use animal models to achieve our aims. Similar types of studies have been carried out in mouse models within the gut microbiome field and have yielded unique insights that has led to the development of effective new drugs for use in clinical management of intestinal diseases (e.g. for Clostridium difficile infections).

Which non-animal alternatives did you consider for use in this project?

We considered the following alternatives for use in the project:

Use of human lung cells cultured in a test tube in the lab

Use of studies directly in human patients.

Why were they not suitable?

Use of human lung cells cultured in a test tube is an extremely simplistic model that does not accurately recreate the make-up of bacterial communities within the lungs. The immune system is also extremely complex and involves a number of other cells apart from lung cells including a range of different white blood cells that have diverse roles. It is impossible to recreate this complexity in an artificial lung cell culture model and therefore these models are extremely limited for understanding these relationships.

Use of studies directly in human patients is currently unfeasible as it is not possible to deplete the human airway microbiome in the same way we can in mice. We also cannot currently administer bacteria into the lungs of patients without extensive safety testing and ethical approval. Finally, we would not be ethically permitted to block many substances produced by the immune system that may be under control by the microbiome in humans.

Overall, the mouse was the best model to enable us to manipulate the airway microbiome in a predictable way that is still highly relevant to human disease to gain functional insight into how commensal bacteria interact with 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?

This work is supported by research grants where detailed proposal of numbers required to test our hypotheses have been calculated. Estimated numbers are based on experimental/control groups required in the proposed experiments. The numbers have been generated on the anticipation that initial timecourse experiments will be used to define optimal single timepoints to be used in more focussed subsequent studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?
We consulted the 'Prepare' and 'Arrive' guidelines and used the NC3Rs Experimental Design Assistant Tool to ensure that all studies are carefully designed and implemented in line with recognised standards and that all sources of bias are considered and minimised. For testing of novel therapies (e.g. administration of commensal bacteria), a larger control group and smaller test groups will be used which can provide sufficient power whilst reducing animal numbers. For dose finding experiments, we use small numbers of animals. Initially, a timecourse experiment is usually conducted. Subsequent experiments are then carried out at a single timepoint where the greatest effect/change was observed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All animal numbers and costings for this proposal were calculated in consultation with the Animal Facility Manager, according to the 3Rs. The number of animals per group will be reviewed regularly throughout the project to determine if protocol variability would allow a reduction in numbers while still providing meaningful results. For testing of novel therapies (e.g. administration of commensal bacteria), a larger control group and smaller test groups

will be used which can provide sufficient power whilst reducing animal numbers. For dose finding experiments, we use small numbers of animals in pilot studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are adopting fully optimised and validated mouse models of viral, bacterial or fungal infection that mimic the key features of human lung disease +/- respiratory infection. Mouse experimental infection models used in this project have been used widely in respiratory research for many years and therefore we can be confident that the methods used cause minimal pain, suffering and distress whilst providing meaningful results that are closely relevant to understanding of human disease.

Why can’t you use animals that are less sentient?

The mouse is the most appropriate animal for understanding host-microbial interactions for a number of reasons. It is an organism that is closely related to humans with structurally and functionally related genes and close similarities in many aspects of how the immune system acts. Relevant resources to block or boost certain substances (e.g antibodies) released by the immune system are also widely available for use in mice. This allows us to carry out functional experiments to manipulate components of the immune system to directly understand whether they play a causal role in disease. These types of approaches are less readily available in other animals. Use of this species for these experiments also offers the benefit of a vast amount of existing literature ensuring that protocols are refined and animal welfare can be better ensured than other species where the same level of understanding is not available. Use of less sentient animals would not be suitable for this project as we require a model which mimics the response of the human immune system to infection and a system where we can reliably block specific immune mediators that we believe to be important. The mouse is by far the best model to achieve this.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will always make sure that, by surveying the scientific literature, studies are not unnecessarily repeated. Appropriate negative and positive controls for a given treatment are essential to avoid unnecessary repetition of experiments, but where pilot experiments demonstrate that a control is redundant, study designs will be refined accordingly in future studies.

We will also keep up to date with latest advances/optimisations of experimental animal models of COPD and asthma through reading the literature and conference attendance to ensure that the most refined models are being adopted. As an example, we ourselves have previously refined a 4 week dosing protocol to induce COPD down to a single dose.

Such optimised approaches can reduce the number of anaesthesia administrations required per animal.

Animal suffering will be minimised by regular checking of animals for relevant symptoms that constitute the end point of the experiment.

In some experiments, we will carry out lung function measurements. We will use unrestrained whole body plethysmography (our normal approach) which is a mild procedure that does not require anaesthesia and allow mice to be utilised for further analyses subsequent to lung function measurement. Only where differences between study groups are deemed significant will we progress to invasive assessment on anaesthetised mice. This approach constitutes both a reduction and a refinement in the use of terminal procedures in line with the 3Rs directive. Good, sympathetic animal handling techniques will be used throughout to minimize discomfort.

The use of germ-free mice, where appropriate, will help reduce animal usage as there is less variation between mice in an experimental group, in comparison to antibiotic treated mice. Thus it is often possible to use fewer mice per experimental group and therefore fewer types of experiment are required to demonstrate that the microbiota is causing a particular outcome in the animal, thus reducing the total number of animals required to confirm or refute the research hypothesis

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be designed in accordance to the NC3Rs PREPARE and ARRIVE guidelines. The models described have been optimised over a number of years by us and others in the field and represent the most refined approaches recognised currently. We will continue to consult publications, guidelines and attend relevant conferences to keep updated and will refine our methods where needed to ensure we keep up to date with the field standards.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will proactively seek to stay informed about advances in 3Rs in order to implement these during the duration of this project. All personal licence holders working under this licence will attend the ‘Guide to the 3Rs & Responsible Animal Research’ course run locally. This course will serve as an introduction to the concept of 3Rs and provide a solid foundation in principles related to implementation of 3Rs, animal welfare and standards for good practice. We will regularly liaise with our dedicated 3Rs programme manager who oversees educational activities related to the application of the 3Rs principles to animal research across the University. We will participate in Animal Welfare and Ethical Review Body (AWERB) meetings locally to ensure we remain up to date with local developments. We will additionally attend internal 3Rs training courses and seminars including in vivo statistics courses with an emphasis of 3Rs. We will also attend training days and seminars run by external organisations such as the NC3Rs and the Fund for the Replacement of Animals in Medical Experiments (FRAME).

Finally, we are planning to start a monthly 3Rs journal club which will focus on a paper selected from the NC3Rs F1000 Research Gateway and all postgraduate students within the wider department will be invited to attend and participate. This will allow us to collectively maintain awareness of new developments in 3Rs methods.

92. Neural mechanism of pain and itch in the spinal cord

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pain, Itch, Electrophysiology, Projection neurons, Primary afferents

Animal types	Life stages
Mice	pregnant, adult, 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?

The spinal cord plays an important role in carrying and modifying sensory information perceived as pain or itch, but we still know little about how this information is processed. The aim of this project is to identify populations of nerve cells in the spinal cord and show how these are organised into circuits that underlie these sensations under normal conditions and in disease 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?

Pain and itch are considered to be transmitted by similar pathways. However, there is some evidence that these two sensations are processed by different mechanisms. In fact, pain treatment has no effect on the treatment of itch. We will investigate acute pain and

itch mechanisms in the spinal cord and compare how those signals are discriminated and conveyed to the brain. We will also investigate long- lasting (chronic) pain and itch mechanisms which are more relevant to diseases that need clinical treatment.

What outputs do you think you will see at the end of this project?

Pain and itch are unpleasant sensations caused by various diseases. They reduce the quality of life and represent a significant economic and societal burden. However, the treatments for these conditions, especially itch, are limited because the mechanisms by which pain signals are transmitted within the nervous system are still largely unknown. A better understanding of these pathways will allow the development of new therapies that are more effective, more selective and have fewer undesirable side- effects. Outputs will include publications in peer-reviewed open access journals.

Who or what will benefit from these outputs, and how?

This study will provide fundamental understanding of the circuits for pain and itch in the spinal cord. Although this will not result directly in development of new treatments in the short term, the improved knowledge provided by this project should lead in the longer term to development of new forms of treatment for chronic pain and itch, and will therefore eventually be beneficial for patients.

How will you look to maximise the outputs of this 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: 10,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice because of the availability of genetically-modified animals that allow specific types of nerve cell to be identified and have their functions altered. An additional advantage is that models for pain and itch have already been established in this species. 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.

Typically, what will be done to an animal used in your project?

Some animals will undergo a chronic pain model, involving either chemotherapy treatment or nerve injury.

Chemotherapy treatment neuropathic pain model: We will inject drugs that are used for chemotherapy in cancer patients, such as paclitaxel. These will be administered up to a maximum of four times over a ten day period. This will lead to a persistent pain state, similar to that seen in patients treated with these drugs, in which there is increased sensitivity to mechanical stimulation and to changes in temperature.

Nerve injury neuropathic pain model: We will perform surgery on nerves in the hindlimb that convey skin sensation resulting in a long-term change that mimics neuropathic pain in patients. Again, this will result in increased sensitivity to mechanical stimulation and to changes in temperature.

In both of these models, acute pain thresholds will be tested by using normally non-painful mechanical and thermal stimuli from which the animal can readily escape. These sets of behavioural tests will typically be performed on three occasions for each animal. We will also observe any spontaneous behaviours, including licking, flinching and wiping of the skin. Animals will be on procedure for no more than 8 weeks after induction of the neuropathic state.

Some of the animals will undergo a chronic itch model. We will topically apply irritants on 5 days a week for a maximum of 3 weeks. This will result in a persistent increase in itch on the affected skin region. We will assess spontaneous itch by observing behaviour such as scratching and biting. We will assess evoked itch by recording scratching or biting behaviour induced by normally non-painful mechanical stimulation or by application to the skin of chemicals that cause itch. Animals will survive for no more than 8 weeks after induction of the chronic itch condition.

Some of the animals will receive injections into the spinal cord or the brain of chemicals or non-toxic viruses. These will allow specific nerve cells to be labelled with fluorescent markers or to have their activity manipulated. In most cases, these injections will be performed on normal animals, but in some cases (estimated 25%) they will be carried out on animals that also undergo either a chronic pain or itch model.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that receive injections into the spinal cord or brain 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.

Neuropathic pain models will show relatively mild signs of pain that include increased sensitivity to mechanical and thermal stimuli, and these effects may be permanent. The animals' ability to move around their cages is not reduced after these procedures, and they eat and drink normally.

The chronic itch model will cause chronic itch conditions such as scratching, which will last for the duration of the experiment. Again, the animals' ability to move around their cages will not be affected, and they will 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 animal type)?

Most of the animals in this study (~70%) will experience only mild severity. The remaining animals (~30%) will experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Sensory mechanisms, including pain and itch, are complex and involve the interaction of multiple types of nerve cells. In the spinal cord, there are various types of cell and their roles are still poorly understood. Although experiments with cultured cells can be used to investigate the function of individual cell types, studies designed to examine 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.

Which non-animal alternatives did you consider for use in this project?

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, and therefore do not allow investigation of how nerve circuits in the spinal cord contribute to pain and itch.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 determine the numbers of animals based on estimated sample size calculated and our previous experiences. When necessary, we will consult statisticians.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Advice was taken from a local statistician and from online tools for performing power calculations. We will also use the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding of genetically modified mice will involve keeping animals that have two copies of the mutation whenever possible. This will reduce the numbers that need to be generated to obtain sufficient mice of appropriate genetic types.

For behavioural experiments, we will perform pilot experiments that will allow us to estimate the minimum number of mice to be tested to produce statistically significant results. For experiments involving recording of nerve cell activity we will record from several different nerve cells in a single spinal cord, and test multiple stimuli on each cell, in order to reduce the number of experiments required. Where possible, we will use gene-altered mice to label specific population of spinal cord cells, thus avoiding the need to use brain injections in order to reveal these cells.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use nerve injury and chemotherapy models to investigate chronic pain, and a model that resembles eczema to investigate chronic itch. These models are all well-established and widely used in pain and itch 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, apart from increased scratching and biting of the skin in the itch model. All animals will be monitored regularly using monitoring sheets.

Why can't you use animals that are less sentient?

Mice are the lowest animals, in terms of evolution, on which these experiments could be conducted. They 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. The majority of 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.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All the animals will be humanely killed at the end of the experiments. All surgical operations will be performed under general anaesthesia. Any clinical problems will be dealt with in consultation with the NVS. There are strict pre-determined ‘humane endpoints’ in place, based on clinical signs, at which animals are promptly and humanely killed to ensure that no animals suffer unnecessarily.

What published best practice guidance will you follow to ensure experiments are conducted in the 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 stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website and elsewhere).

93. Investigating the impact of different management approaches on laboratory animal welfare using objective measures of affective state

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

refinement, affective state, animal welfare, behaviour, husbandry

Animal types	Life stages
Mice	adult, juvenile
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve the objective assessment of affective state in non-human species and apply these methods to better understand the welfare implications of different housing and husbandry procedures for laboratory 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.

Why is it important to undertake this work?

Animals housed in captivity are restricted in terms of expressing natural behaviours and this can lead to poor welfare. Provision of food and water is essential for survival but housing and husbandry procedures impact of the animals' wellbeing and affective state. It is unlikely that any human-managed environment can fully replicate the animal's natural state and deciding on optimal housing and husbandry methods within practical constraints

has largely been based on a human perspective of the animals' experience and are not based on objective assessment of the animals' affective experience.

The majority of current research relies on indirect measures such as overt changes in behaviour or stress-related physiology. We have developed more reliable and unbiased methods to assess affective state in laboratory rodents and now seek to apply these to reduce the cumulative suffering in laboratory rodents which arise from different housing and husbandry methods and refine simple procedures. Our methods are highly specialised and not easily used by other researchers and so a second component of this project is to use our objective readout of affective state to provide a ground truth against which to assess whether there are indicators of a change in affective state e.g. ultrasonic vocalisation, which are easier to record and more widely applicable.

What outputs do you think you will see at the end of this project?

The primary outcome from this project will be data, based on affective state measurements e.g. by assessing the animals performance in behavioural tasks that quantify either decision-making (judgement bias tasks) or learning and memory (affective bias test and reward learning assay), to indicate the changes to the housing and husbandry systems of laboratory rodent required to bring about improvements in animal welfare.

We will also undertake studies to further develop behavioural tasks and measures which can be used to objectively quantify affective state in rodents with the aim to improve their ease of use and hence wider applications.

Who or what will benefit from these outputs, and how?

Within its 5 year time scale, the project is expected to generate empirical data identifying changes to housing and husbandry systems used for laboratory rodents required to bring about improved welfare. In so doing, the study will provide the scientific evidence needed to support changes in policy which benefit animal welfare. The study findings will be relevant to all research institutions managing laboratory rodents including academia, industry and breeding establishments.

The data generated from the studies developing improved methods to objectively assess affective state could offer the potential for larger scale assessments of different housing and husbandry methods enabling other research groups to undertake their own assessment of their animal management and procedures. These methods will benefit both welfare researchers and scientists interested in emotional behaviour and the development of novel treatments for human mood disorders. With the validation work we have planned in this project, these methods could also replace less refined approaches.

In the longer-term, the outcomes of this work will be to generate the empirical data required to support changes in policy.

How will you look to maximise the outputs of this work?

Our aim is to always publish all the results from our studies. We utilise pre-registration and support open science using pre-print servers to disseminate completed studies as soon as possible.

The methods we have developed and specialist expertise in translational rodent models for psychiatry research also means we have built an excellent network of collaborators in

both academia and industry. Importantly, we are currently supported by funding from the NC3Rs which supports researchers in dissemination through specialist publications and their own website provides a platform for researchers to access the latest 3Rs developments. Our excellent networks across academia and industry will also support disseminating our findings and maximising the impacts for arising outputs.

Species and numbers of animals expected to be used

- Mice: 1200
- Rats: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our objectives are specifically related to the welfare of laboratory rats and mice, the most commonly used laboratory species, and therefore we need to use both these species to achieve our objectives. There are species specific differences in the optimal management approaches for mice versus rats and hence we will need to use both species. The choice of species for the specific study will be determined by the experimental objective and some studies may involve comparisons between mice and rats.

As our objectives relate to the impacts of housing and husbandry on animal's lifetime experience it is important that we can replicate the experience of laboratory rodents at different stages of the life course. Most studies will focus on young adults which are the age range used most widely but we also want to understand the longer term impacts on animals and so some studies will work with juvenile animals through to a maximum of 15 months old.

Typically, what will be done to an animal used in your project?

Animals may be exposed to different housing or husbandry methods e.g. group versus individual housing, different types of environmental enrichment or handling procedures. We then use behavioural measures from specially designed reward-based tasks to directly compare the affective state consequences of different management methods.

The studies aim to enhance animal welfare and so will utilise current standard husbandry methods and compare these with approaches designed to induce more positive affective states. For some studies, this may include housing and handling of animals using methods which are hypothesised to be less refined but we need to be able to show this using our objective methods. Although we will manipulate the cage environment, animals will always be provided with nesting material as a minimum. Some animals may be housed individually for periods of up to 6 months.

In studies where we are testing the validity of a method to quantify affective state, animals may undergo acute or chronic manipulations designed to induce relatively more positive or negative affective states using either pharmacological or psychosocial manipulations. The pharmacological manipulations will use refined dosing methods with most treatments administered using drinking water or in palatable solutions which are self-administered by

the animal. For some treatments we may need to administer by injection with a brief period of restraint (mice only) or using our refined method (rats). Animals may receive repeated treatments, by the most refined route for the pharmacokinetics of the drug, up to once daily and for periods of up to 3 months. Psychosocial manipulations may include acute or chronic exposure to individual housing, altered housing density and exposure to different animals of the same species for periods up to 3 months.

A typical experiment will last ~6 months and will include pre-training of animals in the behavioural task which may include food or water restriction to motivate behaviour. Animals undergo a period of housing and/or handing manipulation and then are re-tested in the behavioural tests before they are killed and tissue and blood samples (max of 4 tail bleeds) collected for post mortem analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals used in these studies will not show any clinical signs or experience procedures which involve more than a transient period of acute pain or distress. The majority of animals will experience housing and husbandry conditions which will be similar to those used under standard laboratory conditions.
Animals may experience increased levels of inter-individual aggression, long term negative affective states and/or short-term stress from the pharmacological and psychosocial manipulations. For some studies animals will require food or water restriction to motivate their engagement with the rewards available in the behavioural task. Restriction will cause mild hunger, but animals will maintain growth and restriction is only used when animals are doing the behavioural tasks and they are returned to ad libitum the rest of time they are in the protocol.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice = mild, 100% Rats = mild, 100%

What will happen to animals at the end of this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our primary interest is in the welfare outcomes of different husbandry systems management methods and this can only be achieved using intact living animals of the relevant species which will benefit from the outcomes. We are also studying complex, higher order behaviours which are only expressed in the more complex mammalian

species and cannot be modelled in lower species. To achieve the aims of our project we need to be able to undertake direct manipulations of the animals housing and husbandry experience and then quantify the arising affective states.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are not suitable for this project.

Why were they not suitable?

There are no non-animals alternatives which could be used in this project as the scientific objectives are species specific and involve assessing the affective state, which only exists in higher living organisms like mammals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated based on our current research funding and the specific objectives defined in those research grants. We have also included some animals based on our projected research income and projects which are currently in development or under review with funding bodies. The studies are expected to evaluate ~ 3 variations in housing in conventional versus IVC caging (NC3Rs grant) and 6 studies looking at variations in husbandry and handling techniques using a group size of 12-16 cages. The sample size is determined from data generated during previous work. Evaluation of novel measures of affective state will use group sizes of 16-24 cages based on our pilot data and will investigate ~2 different behavioural assays and 4 studies focusing on home cage recording monitoring of behaviour and ultrasonic vocalisations.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have undertaken meta-analyses of the behavioural methods to provide a reliable estimate of effect size and then used this to design our experiments. Where required, we integrate both the behavioural work and ex vivo studies to maximise the data we can obtained from any one animal and enhance the interpretation of the behavioural studies. For relatively simple studies we can utilise the NC3Rs EDA but where we require more complex designs, we work with local experts in experiment design or our industrial partners who have dedicated statistical support within their organisations.

Depending on the specific objective where possible, we utilise within-subject study designs. This reduces the total numbers of animals as each animal can act as its own control and generate multiple data points with reduced variability. Although this does mean animals may be on protocol for longer or receive more interventions, combined with our refinements, these should not lead to any significant increase in cumulative suffering but can dramatically reduce animal numbers.

When we are working with chronic manipulations, within-subject designs are not appropriate and for these studies we reduce the numbers of animals by limiting the use of placebo controls and utilise historical data wherever possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We collaborate with computational neuroscientists to maximise the outputs from our behavioural data.

The same animal may be used across a number of behavioural tasks so we can look at individual phenotypes and understand how changes in affective state affect the wider behavioural profile of the animals. This avoids the need to use separate cohorts for each behavioural test and also reduces variability and overall numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of studies will compare current housing and husbandry methods with approaches designed to be more refined (~70%). Our current focus is on housing of male mice and the extent to which inter-cage aggression leads to poor welfare versus individual housing and how the affective state of animals in group versus individual housing is affected when they are housed in conventional versus individually ventilated cages. We are also interested in the impacts of temperature and whether mice are in a relatively more positive affective state when housed at higher temperatures and how this interacts with group versus individual housing. Our rat studies focus on the welfare benefits of caging which can increase available space particularly enabling more vertical behaviours and whether play pens and human simulated play can generate more positive affective states. This will mean that some animals may be exposed to management methods which are hypothesised to be more stressful and/or less optimal e.g. different types and levels of environmental enrichment, different handling methods with or without habituation, but we need these comparisons to provide the objective scientific data to show welfare benefits and impact on policy.

For studies designed to develop better methods to objectively quantify affective state, some animals will experience short term or long term negative affective states generated using mild interventions. These are necessary to validate the methods and it is important to be able to compare with established literature and to determine if the readouts are generalisable or only sensitive to a specific type of manipulation. To be of widespread applicability, these readouts need to be reliable and sensitive across different types of manipulation and so it is important that we compare different models. The affective state manipulations we propose using are expected to induce affective state changes similar to

those animals might experience within the normal routines of animal management e.g. stress associated with short periods of restraint for health checks.

Why can't you use animals that are less sentient?

The scientific objectives of the project are specific to rats and mice and cannot be undertaken in a less sentient species and require an awake behaving animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals undergo a minimum of one week's habituation to handling which is combined with rewards to enhance the animal's positive association with human interactions. Non-aversive handling methods are used, and animals are housed in enriched cages unless the specific scientific objective requires a different approach i.e. when comparing the welfare implications of different handling methods.

Substance administration will use refined methods with animals trained to take drugs from a syringe in a palatable solution or using modified restraint method which we have shown reduces stress responses by ~50%. Only where the pharmacology of the treatment does not permit one of these refined routes would an animal be treated using a less refined method.

Inductions of a negative affective state will use the least aversive method and duration which can generate the behavioural response we require or is necessary to re-produce the housing or husbandry condition.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have been leaders in the field of refinements for cognitive and affective neuroscience and utilise our objective methods to quantify emotional state in rodents and disseminate our findings through publications and presentations.

I attend regular CPD events including those run by the NC3Rs and assess and implement refinements which are compatible with our research objective. I attend meetings such as LASA and subject specific conferences. I also utlise the NC3Rs resources to follow the latest guidance on procedures including methods to recognise and manage suffering.

Studies are designed and reported in line with both the ARRIVE and PREPARE guidelines and all data are made freely available following publication.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I attend the annual 3Rs symposium held across the local University network and regularly attend webinars and workshops hosted by the NC3Rs. I attend and contribute to national and international conferences in my field enabling me to keep up with the latest developments and also disseminate outcomes and refinements from our own research programmes. The institution also benefits from an NC3Rs regional manager with whom we have established an excellent working relationship and regular meet to discuss regional matters relevant to our research and the 3Rs.The development of refined methods has been a core objective for my research and is being implemented in this application.

94. Mononuclear phagocyte function in inflammation and cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

immunity, mononuclear phagocytes, cancer, inflammation

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this project is to understand how white blood cells called mononuclear phagocytes participate in the control of cancer and the response of patients to drugs that are designed to activate white blood cells to kill cancer cells. We will also study the roles of these cells in the unwanted side effects of these drugs, which can often result in patients suffering from inflammatory diseases such as inflammatory bowel disease (colitis) and arthritis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Improved understanding of the genetic basis underlying mononuclear phagocyte regulation of immunity in the context of cancer and immunotherapy which may lead to the development of new improved therapeutic approaches.

What outputs do you think you will see at the end of this project?

The goal of this project is to understand how macrophages (MF) and dendritic cells (DC), which develop from white blood cells called mononuclear phagocytes, help provide protective immunity against cancer cells and immunotherapy. Specifically, we want to understand how we can target these cells to improve anti-cancer immunotherapy and reduce unwanted side effects. Given the tissue specific nature of the tumour microenvironment, it is important to address these questions in a range of tumour models to assess common and unique aspects of MF and DC function in cancer immunotherapy.

Who or what will benefit from these outputs, and how?

Improved understanding of the genetic basis underlying MF and DC regulation of immunity in the context of inflammation, infection and cancer which may lead to the development of new approaches to vaccination or immunotherapy.

How will you look to maximise the outputs of this work?

This project will involve several national and international collaborations. Results of the research and methodological developments will be disseminated at national and international conferences and in peer reviewed publications in the scientific press.

Species and numbers of animals expected to be used

- Mice: 24100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Use of laboratory mice provides a unique way to test and analyse tumour growth and immune responses a) in realistic in vivo conditions, b) in models in which genetic conservations bring knowledge relevant for the human immune system, c) in a genetically controlled system. Mouse experiments have and continue to contribute to the development and testing of new anti-cancer treatments, vaccines and immunotherapies in patients and improving human health.

Typically, what will be done to an animal used in your project?

We intend to understand how different types of mononuclear phagocyte populations affect tumour development and responses to immunotherapy, including adverse autoimmune events associated with therapy such as arthritis and colitis. We will use laboratory mice, including genetically modified strains bearing modified gene loci relevant to mononuclear phagocyte function. Mice will typically be injected with different tumour cells or inflammatory stimuli to model tumour growth in different organs for example the mammary gland, pancreas or peritoneal cavity and we will measure the effects of specific genes in the immune response to specific antigens expressed by the tumour cells. Typically we will measure effects by imaging immune cell behaviour in tissues of using flow cytometry and imaging.

What are the expected impacts and/or adverse effects for the animals during your project?

Immunisation:

Animals may experience slight discomfort associated with inflammation similar to mild, self-resolving infections or vaccination in humans that resolves in several days.

Irradiation:

May cause damage to gut epithelium resulting in malabsorption and weight loss, and allow overgrowth of microorganisms resulting in enteritis. Leukopenia may cause bone marrow depression resulting in increased susceptibility to infections, platelet deficiency may increase risk of haemorrhages and anaemia. These are only likely to occur in the rare instance that reconstitution with donor cells fails.

Tumour growth:

Strong immune responses against soft tissue tumours may show signs of ulceration, which may be suppurating or exudative. In rare cases, tumour growth may result in systemic adverse effects associated with inflammation or weight loss.

Tumour growth in the peritoneal cavity or pancreas may result in a marked abdominal distension produced by peritoneal ascitic fluid. Intra-venous injection of tumour cells can cause dyspnoea due to lung metastasis.

Spontaneous tumour growth in the mammary gland, lung, epidermis and pancreas is usually well tolerated. Excessive tumour growth may lead to general signs of distress or pain; sensitivity to handling, pilo-erection or persistent hunched posture, other clinical signs suggestive of excessive tumour burden include weight loss, impaired mobility.

Surgery:

After surgery, wound breakdown may be observed. Colitis:
Animals may exhibit weight loss and diarrhoea, weight should remain in the region of 85-95 % of initial weight before spontaneous full recovery (from around 5-7 days).

Arthritis:

Mice will show swelling and reddening of one or more paws and reduced mobility. In very rare cases (<1%) ulceration may occur. Severity normally reaches a maximum at around 10-14 days after onset and spontaneously resolves between 21-28 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?
The majority of protocols involve acute inflammation that resolves in several days and are of mild severity (70 %). Tumour growth experiments, colitis and antibody-induced arthritis will be considered moderate (30 %) due to their longer duration (typically <30 days).

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?

Mouse models enable genetically controlled experiments that can lead to the establishment of causal relationships, thus permitting more conclusive experiments to probe the functions of specific genes in the immune system. The high level of conservation between genes, cell types and signalling pathways means that findings acquired in the mouse model are generally highly relevant for our understanding of the human immune system and relevant diseases, including infectious diseases, autoimmune diseases and cancer.

Which non-animal alternatives did you consider for use in this project?

Besides our use of in vivo animal experimentation, our lab has developed a numbers of primary cell culture methods for the analysis of specific cell interactions in vitro and the development of alternative methods for the production of genetically modified MF and DC using stem cell cultures (iPSC). These approaches are used in parallel to help reduce our involvement in animal experimentation within the frame of our scientific objectives. For example, some target genes may be screened in vitro first for their effects on MF or DC activation in order to limit the number of GM mouse strains that would be evaluated in vivo.

Why were they not suitable?

The development of adaptive immunity is a complex process involving the interactions of different cell types such as MF, DC, T and B lymphocytes, and the activation of multiple signalling pathways. These interactions occur in specific tissue microenvironments created within the highly organised areas of lymphoid organs. In addition, immune responses are tightly controlled by modulation of HSCs in the bone marrow providing MF and DC progenitors that migrate from the blood to peripheral tissues and lymphoid organs. As a result, it is impossible to model all these interactions by in vitro studies only.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Extensive experience with the models proposed has shown that 6-8 animals per group is usually sufficient to obtain statistically robust data. Some procedures are expected to be of moderate severity (protocols 7-9) and intervention to minimise potential suffering will be required. Thus, group size needs to account for this and remaining animals sufficient to

provide statistical significance. Statistical advice is on hand at the within SIMS at KCL to optimise approaches where necessary.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Multi-parametric analysis will be performed on tissues harvested post-mortem in all experiments to maximise the information obtained and reduce the number of mice used and more generally the number of experiments required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When appropriate, surplus tissues will be conserved for the establishment of reference samples and to minimise the need to new experiments.

Efficient GM colony management will prevent unnecessary breeding and minimise offspring with undesirable genotypes. Littermates genotyped as heterozygous or WT will be used as appropriate age and sex matched controls. This allows optimal use of mouse numbers generated and is best scientific practice for the study of genetic alterations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Protocols have been optimised to induce mostly sub-clinical, non-lethal and self-limiting inflammation in C57BL/6, that will be used in this project. These models trigger transient acute local inflammation, which may possibly result in slight, but reversible, discomfort and possibly weight loss.

Models of tumour growth (protocols 5, 7) are required to assess the development of protective immunity against tumour-associated antigens. These studies will aid the rational development of protective anti-tumoural immunotherapies, leading to the activation of CD8+ T cells capable of limiting tumour growth.

Microbial challenge experiments (protocol 3) will utilise non-lethal microbial stimuli in C57BL/6 mice, these protocols are designed to induce a transient acute inflammation that may involve the onset of transient “flu-like” symptoms and mild discomfort, including slight but reversible weight loss.

Why can’t you use animals that are less sentient?

The vast majority of experiments will be performed with C57BL6 mice because this is the strain of choice for genetically modified mice. Adult mice will be used with a fully mature immune system to reflect the relevant clinical contexts.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all procedures, an integrated daily monitoring will be performed. When necessary, analgesia and/or temperature control will be used to alleviate pain, discomfort or distress, due to acute inflammation. Mice will be killed if they show any persistent adverse effects after 24 hours, or weight loss of >20%.

For tumour growth experiments, humane endpoints will be applied rigorously according to established guidelines for experimental tumour growth. Tumour size will be monitored and animals killed at fixed, pre-defined time points before they reach clinical signs (protocols 5, 7).

In microbial challenge experiments (protocol 3), animals that fail to overcome infection will be killed by a schedule 1 method.

To ensure the rigorous implementation of humane endpoints, all experiments will be performed by specifically-trained scientists, holders of personal licenses and expert in the implementations of these models.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Smith AJ, et al. (2018) PREPARE: guidelines for planning animal research and testing. Lab Animal 52(2):135-141.

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156.

Workman et al. Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer (2010) 102, 1555 – 1577.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We subscribe to the NC3Rs monthly updates on the latest science and technology to replace animal studies, providing information new research approaches that may avoid the need for in vivo models. NC3Rs resources also help support best experimental design to ensure robust and reproducible methods and outcomes, as well as guidance on the latest knowledge to improve laboratory animal welfare.

95. Fish developmental biology 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

development, stem cells, tissue homeostasis, congenital disease, ageing

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult
Medaka (Oryzias latipes)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the cellular, genetic and molecular mechanisms of development and stem cell function. We also want to develop fish models for human congenital conditions in order to understand better the basis for these conditions and to allow screening for candidate drug 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?

There are two main reasons to do this work. The first is simply to understand a basic aspect of developmental biology - how a complex, multicellular organism is formed, and maintained. The second derives from the fact that these same processes occur in all

organisms, including humans, and so knowledge of these mechanisms underpins our ability to understand and diagnose, as well as perhaps to treat, human congenital disease (e.g. Hirschsprung's disease and other neurocristopathies), but also human ageing, since these stem cells are the source of new cells for tissue maintenance.

What outputs do you think you will see at the end of this project?

Our work will result in outputs through the appropriate channels of conference presentation and publication (20+ and 10+ respectively), wherever possible in high impact and/or open access journals; in addition, we anticipate generating models of human congenital disease (e.g. multiple (2-5) humanised mutants for *sox10*, expected to allow modeling of SOX10-dependent neurocristopathies). These outputs will be freely accessible to other researchers, clinicians and industry. These end users will be able to utilise them in developing their own research and biomedical applications.

Who or what will benefit from these outputs, and how?

In general, the short-term (1-5 yrs) benefits will be to our and other academic, clinical and biomedical industry researchers, by providing increased understanding of the molecular genetic mechanisms of normal neural crest cell development, and thus of what goes wrong (e.g. better genotype-phenotype correlation) in the context of congenital diseases of the neural crest (neurocristopathies), particularly Hirschsprung's disease (prevalence 1:5000) and Waardenburg syndromes 1-4 (all of lower incidence). In the medium/long-term (5-15 yrs) we anticipate our findings and approaches may influence perception, diagnosis and perhaps even treatment of these diseases.

How will you look to maximise the outputs of this work?

We will disseminate our knowledge through publication and presentation at conferences and in institutional seminars: We have an excellent track record of publication (e.g. 1 book (editor), 5 Book Chapters, 25 journal articles/commentaries published during period of previous license) and visibility (our work has been cited >15500 times, including >4450 since 2016 (Google Scholar, accessed 10/11/2021)). In addition, we regularly present our findings at major international, national and local conferences and through institutional seminars (e.g. I have given 63 (10 during period of previous license, despite the massive impact of COVID) invited conference presentations, plus a further 17 (3) meeting talks as selected abstracts, 113 (20) poster presentations, and 82 (15) invited institutional seminars). Through contacts established over the years we have been able to build a broad and expanding network of national and international collaborators; currently active collaborations expected to continue into the current license period include those with researchers in Bristol, Exeter, London and Surrey, plus the USA, Russia, Japan and France, but we will also be open to developing new collaborations wherever they will maximise our outputs. We will establish collaborations with other researchers by sharing knowledge, experience and reagents. We will also look for opportunities to work closely with clinicians and industry optimizing the chance of translational impact. Publication of unsuccessful approaches is a new opportunity and one we will be open to exploring if/when appropriate.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 33800
- Medaka (Oryzias latipes): 3250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

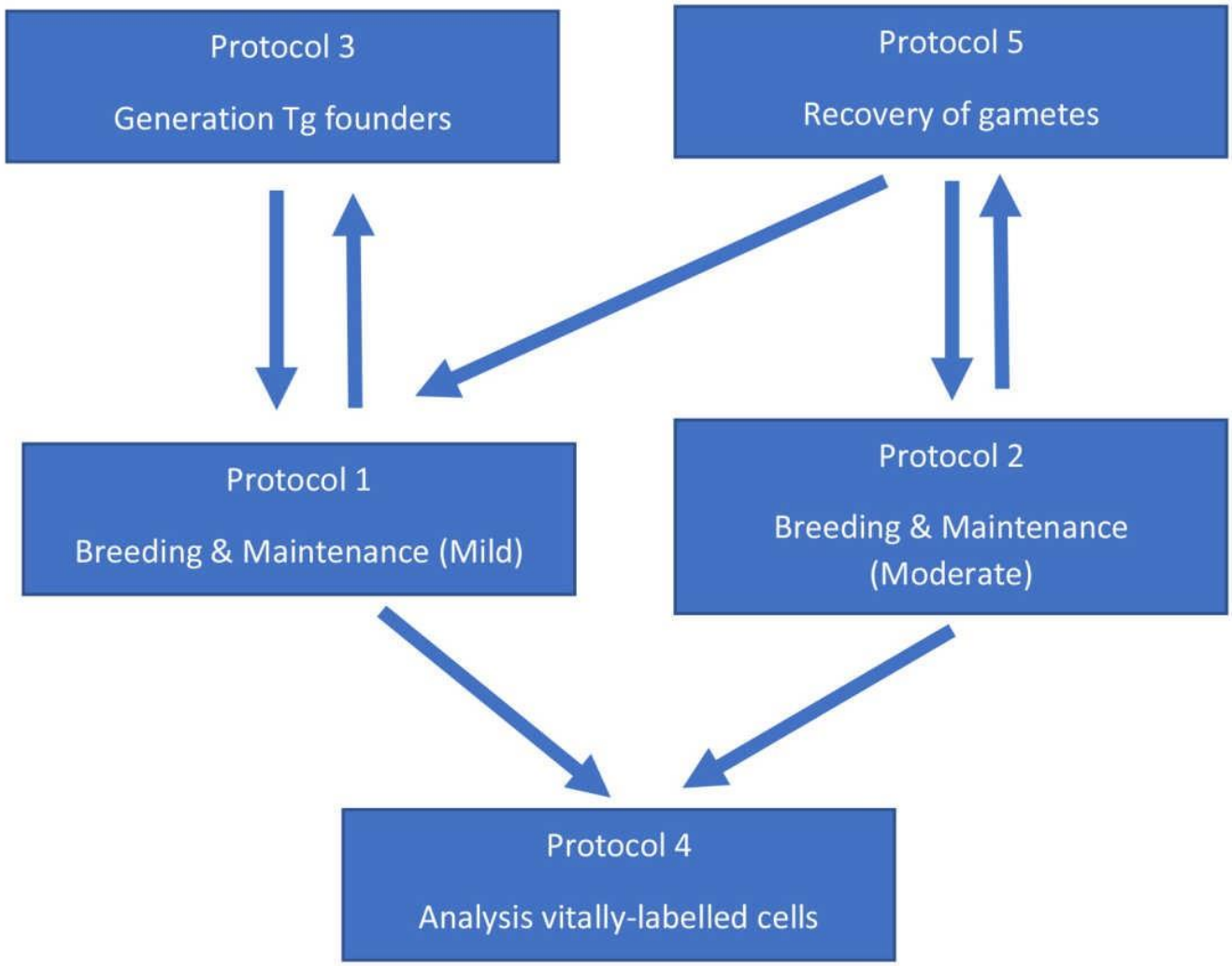
Explain why you are using these types of animals and your choice of life stages.

We use fish model systems since these are the least sentient of the vertebrate model systems, but also because they provide significant experimental advantages. Zebrafish and medaka are the two best- studied fish model systems, and thus have an abundance of resources facilitating their experimental investigation. The animals develop externally and are optically clear (often allowing for observation of development with no intervention needed); we therefore make most of our observations without any need for surgery or other intervention giving discomfort to the protected animal. Our work largely focuses on embryonic and early larval stages prior to free-feeding, both to minimise the suffering, and to maximise the productivity, since these stages are both appropriate for most of our studies and easiest to work with experimentally. For the pigment pattern work we have to use the juvenile-adult stages since these are when the well-studied adult pigment pattern is being formed, but again observations can be made on intact living animals. For the humanised *sox10* mutant studies it is conceivable that phenotypes will not be detectable until post-free-feeding stages, so we expect to monitor these where scientifically justified.

Typically, what will be done to an animal used in your project?

An animal used in this Project will typically undergo or have previously undergone genetic modification. This is important, for example, for generating fluorescent reporter transgenic lines essential for monitoring the cell behaviour and function, and also for introducing mutations in genes known or expected to influence development. This is an important part of our research in understanding the molecular and cellular mechanisms underpinning development and associated human disease.
Usually fish generated will simply be used to breed embryos for analysis prior to free-feeding. In some cases these embryos will need to be raised to larval or later stages, but usually then will either be fixed for analysis or imaged live under Protocol 4.

Fig. 1 Flow diagram showing transfer between protocols



What are the expected impacts and/or adverse effects for the animals during your project?

Animals used in this Project are expected to experience minimal pain and suffering as a result of our manipulations. However, in certain cases when a genetically altered animal is created to mimic a human disease condition, it is possible that fish might experience some clinical symptoms. This can generally be avoided by maintaining our genetically altered lines in healthy heterozygous forms.

Occasionally, our experimental manipulations can also produce side effects. We will make sure that any individual showing signs of non-transient discomfort is killed immediately by Schedule 1 methods.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority (>90%) of animals used in this Project will experience pain or distress that is no greater than mild. We anticipate that a maximum of <10% of animals used in this Project will experience a moderate severity. Any such individual presenting any signs of pain or distress that is not rapidly curable will be killed under Schedule 1.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

As the main goal of our research is the understanding of the cellular and molecular mechanisms required for stem cell development in the vertebrate embryo, alternatives not using animals at all are not yet available. The originality and strength of our study is the ability to follow development in vivo, using imaging of fluorescent transgenic zebrafish, ensuring that the cellular environment is an authentic one. In vivo studies are the only way to identify the cellular behaviour underpinning developmental defects. Moreover, our in vivo studies allow the examination of the cell behavioural changes resulting from genetic lesions, allowing us to perform small molecule drug screening to identify compounds that could ameliorate the symptoms, offering mechanistic insight and, perhaps, opportunities for novel drug development.

Which non-animal alternatives did you consider for use in this project?

We have been actively exploring mathematical modelling, and have used it extensively in our work on both developmental gene function and pigment pattern formation. In the latter case, we have now published models that mimic the patterning process rather well, and can be used to focus future experimental studies, including those proposed in this project. We have also considered cell culture models.

Why were they not suitable?

The complex environment of the embryo, with multiple cell-types and with cells moving locations in a reproducible way, makes cell culture models (including complex *in vitro* models which do not mimic the complex *in vivo* environment and, as summarised on the NC3Rs website, are an improvement on 2D cell culture, but have significant reproducibility issues) inadequate. However, this is a rapidly changing area, so we will continue to monitor the literature for suitable models 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?

Estimation of fish usage is non-trivial, since multiple confounding factors make simple calculations impossible, and instead force reliance on previous experience within the wider fish researcher community. The key point is that the overwhelming majority of our animal usage is in generating or maintaining genetically altered (GA) lines. In the first case, stochastic effects are important in affecting i) how many founder fish actually have germ-line integration of the transgene, and then transmit to the next generation, as well as ii) the extent to which positional effects alter the behaviour of the transgene from that intended. In both cases, a further stochastic effect that is of major significance is that of the unpredictability of sex ratios. Furthermore, for almost all work, the key issue is a regular supply of good quality embryos for experimental study; this necessitates maintaining larger than minimal numbers of fish so that individuals are not usually used more than once per week. Over-usage would be problematic from a welfare point of view since it could well result in stress. We note that the harmless nature of the GA in the majority of the lines when kept as hemizygotes or heterozygotes goes a long way to reducing to a minimum the suffering caused. The numbers requested in this license for each protocol have been estimated in accordance with these principles.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Key to reducing the numbers being used is that almost all (approximately 90%) manipulations and observations will be performed on zebrafish embryos or early larvae at stages prior to the stage when they are capable of independent feeding, and hence on stages that are outside of the Animal (Scientific Procedures) Act. However, in the remaining cases (approximately 10%) it will be necessary to rear experimentally manipulated fish after they become protected by the Act. Where possible our emphasis will be on assessing effects as early as possible, so that older, larval and adult stages will be only used where completely necessary, and we will take statistical advice to ensure the numbers used are appropriate.

We will take advantage of the optical clarity of fish larvae to allow the use of new microscopic methods to image developing stem cells and congenital disease models. The aim of much of our work is to use transgenes that do not disrupt normal processes to report on those processes as they happen. So, although the animals are transgenic and therefore fall under the Act, they will not suffer in any way compared to nonGA fish. Creating stable transgenic lines is, by itself, a way to reduce the number of animals used. The establishment of healthy viable adults carrying a transgene allows us to reduce the number of embryos studied compared to the quantity we would have to use in a transient transgenic system (i.e. where embryo is injected with transgene, creating a transgenic chimaera), because the transgenic line will be expected to be homogenously expressed within the appropriate cell-types.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most of our animal usage will result from generation and maintenance of mutant and transgenic stocks. We will use the latest transgenesis techniques so as to ensure that the minimum numbers of founder fish are generated, and will take advantage of developments

in inducible gene expression (e.g. CreERT2) to ensure that transgenes are expressed wherever possible only when needed.

We have already used, and will continue to develop, mathematical modelling approaches to limit our experimental work, by guiding us as to the mechanisms that need to be investigated experimentally.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 and medaka as model systems during this Project. Fish are the least sentient of the vertebrate models suitable for genetic research. The Project will use the latest in vivo technologies used by other researchers in the field to minimise any animal suffering, such as well-established gene editing and imaging techniques which allow manipulation of specific genes in selected cell-types and/or during selected time periods, so as to try to avoid systemic or pleiotropic effects. Our methods will allow us to generate transgenic lines reporting the expression in specific cell types of relevant genes, and animals with mutations in genes required for development. We also plan to use imaging techniques to monitor stem cell behaviour and function and assays to investigate the developmental effects of these genetic manipulations.

Why can’t you use animals that are less sentient?

We chose the fish as our vertebrate animal model because these are the least sentient of the major vertebrate models, and because the embryos develop outside the mother (allowing collection without affecting adult mothers) and are optically clear (allowing for observation of development with no intervention needed). We make the vast majority of our observations on embryos/early larvae prior to free-feeding and hence minimise the use of later stages. Live observations of later stages are performed only when scientifically necessary, and by non-invasive methods. We therefore make our observations without any need for surgery or other intervention giving discomfort to the protected animal. We anticipate that the majority of our procedures will not exceed mild severity limit and will not generate pain or distress (a maximum of 10% of animals are expected to exceed that limit, and no animal will experience a severity above moderate). Any individual presenting any signs of pain or distress that is not rapidly resolved will be killed under Schedule 1.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all our studies we will address effects as early as possible and we will use anaesthetics whenever appropriate. Non-invasive methods will be used throughout. As stated in Protocol 1, the majority of lines carrying mutations will be kept as healthy heterozygotes and are expected to only show phenotypes when homozygous. Therefore, this will reduce

any potential suffering of animals. Any individual presenting signs of non-transient distress or pain will be culled under Schedule 1.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will monitor the published literature to ensure experiments are conducted in the most refined way. We will use pilot studies to refine protocols being developed/modified in the lab. We will refine our use of specific anaesthetics as their advantages and disadvantages are understood. Where revised methodology has become available, we will endeavour to update our protocols accordingly.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly interact with our NC3Rs Regional Programme Manager and attend meetings/events organised by the NC3Rs.

96. Mechanisms promoting cancer 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

Key words

Cancer, Immune system, Metabolism, Blood vessel development, Microbiome

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to identify and understand the factors that cause cancer to develop, progress and spread in mice. We will develop and assess new treatments targeting mechanisms driving cancer progression. We will develop novel diagnostic tools to aid identifying key cancer stages.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Breast cancer is the most common cancer in the UK. Colorectal cancer is the third most common cancer in the UK. Unfortunately, these cancers are equally associated with high death rates. For example, only 53% of patient with colorectal cancer survive for more than 10 years.
Early detection of cancers mean patients are more likely to respond to treatment and remain cancer free for a longer period. Previous studies have shown us that different

mechanisms and cell types support cancer cell growths and development.

It is important that we continue to identify these mechanisms and gain better understanding of the range of cell types that drive the development of cancer tumours. This in turn will provide information that will help develop new treatments to target such cells and mechanisms.

What outputs do you think you will see at the end of this project?

Using mice bearing human cancer xenografts and syngeneic mouse tumour models we will have gained the following aspects at the end of this project:

Identification of novel genetic changes in cancer cells that impact on treatments and any resistance to such treatments.

Improved knowledge of how different aspects of the immune system and other cells impact on cancer development and response to treatments

Improved knowledge of cellular responses to low oxygen and nutrient levels.

Use this knowledge to develop new cancer treatments

This information will be shared with the scientific community through publications and conferences.

Who or what will benefit from these outputs, and how?

Patients with solid tumour cancers like breast and colorectal cancers, clinicians and academic researchers will benefit from the outputs of this project.

Ultimately, the results of this research will enable improved diagnostics of patients and also improved outcome following provision to provide more appropriate therapy and increasing current drug efficacy against cancer. This is due to this project enabling us to have a better understanding of the mechanisms and involvement of cell types that add to profile of an ‘aggressive cancer type,’ thus drive cancer progression and resistance to current therapies.

How will you look to maximise the outputs of this work?

This information will be shared with the scientific community through meetings, publications (including unsuccessful approaches involving animals) and conferences. In addition, we hold in vivo meeting regularly and invite collaborators from similar scientific backgrounds to attend and exchange knowledge and experiences. These improve the outputs of our projects.

Species and numbers of animals expected to be used

- Mice: 4200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using mice to model the complex processes in human cancer progression and generate clinically relevant information. The majority of these processes do not occur in non-mammalian species.

Mice need to be at the age that they can bred and produce healthy offspring. Successful growth of cancer and tolerance to therapy is dependent on the use of young adult mice. Juveniles, neonates and pregnant females are used in cases where early deletion of a gene is required, based on the expression profile of that gene.

Typically, what will be done to an animal used in your project?

Genetically altered mice may be bred and given an injection of a gene inducing or deleting agent to modify its genetic status. The administration of these drugs will be typically daily for one week.

Mice may be injected directly with tumour cells or recieve an injection of substances or non-tumour cell types to initiate cancer development. The injection will be given at either the site at which the tumour is normally found or systemically via the tail vein.

Mice may be given a substance or non-proliferating cells to modify the process which is being studied. Administration may be as a single or combination of treatments. Imaging may take place to identify changes that occur at key disease stages.

Typically, samples will be collected at key disease stages to develop biomarkers or therapeutic antibodies and to assess change in the cancer.

What are the expected impacts and/or adverse effects for the animals during your project?

Some mice will undergo tumour implantation under the skin, and these visually observed tumours will grow to a permitted maximum size. Presence of visual tumours near endpoint size may cause some mild to moderate discomfort, such as animals fail to gain weight or have slow weight loss, or have mild diarrhoea. As animals with internal tumour burdens approach endpoint stage, they may experience breathing difficulties if the cancer cells form growths in the lungs or swollen abdomens observed following growth in liver, or gut. Some of the external tumours may naturally ulcerate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold (68%), Mild (18%) and moderate (14%).

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?

Mouse tumour models, in combination with imaging, will allow us to study closely the cellular mechanisms of cancer and the effectiveness of the new diagnostics and therapies. These can be translated to the clinical setting. The animal models of cancer also most closely fit the complex processes and interactions between cell types that occur in human cancer. Some of these processes involving immune cells and other cell types do not occur in non-mammalian species and therefore cannot be studied in lower organisms.

Which non-animal alternatives did you consider for use in this project?

Access to material and results from patients with cancer in the clinic and previous animal experiments are available and will reduce the number of animal experiments required. Collectively, this material and data can be interrogated in a series of computer modelling to aid finding new links to processes and mechanisms driving cancer progression.

We can model certain basic processes in the laboratory setting. Cell culture will be used throughout this project to provide key information on gene function and mechanisms of cell adaptation to specific stresses in culture. We can model basic outcomes following exposure to new therapies (such as reduce growth rates, cause cell death, and block specific blood vessel development stages).

Why were they not suitable?

At present, the use of non-animal methods does not allow us to answer all of our scientific questions. Non-animal models cannot fully recapitulate the 3D nature of the cancer tissue and the many different cell types interacting with each other, the fluctuations in blood delivery of oxygen and nutrients and how these collectively influence response to therapy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 have shown what number of animals are required for each study, appropriate to the cancer type, and conditions investigated with appropriate numbers of animals and controls in the experimental design. Data on the numbers of animals required for breeding and maintaining a productive colony is also available from our archive of mouse database.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We regularly cross-reference our experimental design, where appropriate with online tools such as the NC3Rs Experimental Design Assistant. We also use statistical design models to aid confirmation of appropriate numbers of animals to be used.

Computer modelling of non-animal model data, previous animal experimental data and information from pilot animal studies also aids the selection of appropriate targets and experimental design for maximum output information at the end of the experiment but using the least number of animals. An example of this is incorporating imaging points into our design. Use of imaging increases the output from each animal, creating a better picture of the changes that occur at key cancer progression stages without the need to kill animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Well planned, efficient breeding calculations are carried out to ensure that only those animals needed for colony maintenance and experimental studies are produced. A mouse database gives us the most recent data to enable these calculations to be carried out.

The use of imaging techniques allows for more information to be gathered from each individual animal.

Harvesting of extra tissues and appropriate sample collections at the end of the experiment and banking them allows for future use and negates the need for animals in these cases.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice lacking a functional aspect of the immune system will be used to investigate which part of the immune system influences cancer progression. Such immuno-deficient animals enable us to grow human cancer cell lines that would normally be rejected by an active immune system.
We will use mice with or without a genetic change because we can directly study the mechanisms involved in specific processes in cancer progression, for instance blood vessel development or metabolism changes. Use of genetic modifications enables targeting specific key pathways/mechanisms that is impossible to replace by an alternative method, e.g. due to current lack of appropriate specific inhibitors and avoids animals being subjected to multiple administration of substances.

Animals used for breeding will only undergo methods that are natural and suitable to maintain the genetic status of the strain.

Pilot studies are always conducted using the fewest number of animals to establish the novel growth cancer profile, novel substances used to target cancer progression, and confirm appropriate imaging plan to map changes during tumour progression.

Imaging provides an important insight to cancer progression, enabling us to visualise actual tumour burden and real time changes inside the tumours without dependency on changes in animal health and behaviour.

Why can't you use animals that are less sentient?

The experiments proposed are being conducted in the least sentient possible animal system.

The adult mouse model is relevant to our studies because they enable us to address our studies into processes that promote cancer progression, for example they have a functional active immune system at this stage. The mouse model is most widely used and accepted animal system for these types of studies. In addition, the information provide at this animal stage and tumour models translates into the clinical setting.

Tumour bearing animals and exposure to substances are being conducted in animals that are at an appropriate age, for instance where the immune system is active and initiation of tumour models, blood vessel development is well established.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experimental designs are based on previous recorded and pilot experimental observations and outcomes. This ensures appropriate use of procedures and routes to minimise welfare costs for animals. We provide appropriate pain management following any procedures.

We regularly monitor animals for changes in their health, for example measure their weight change and their behaviour.

Observations during experiments are documented to allow control measures such as increased monitoring or treatments to avoid and reduce further harm to the animals. We always conduct pilot studies to check that measures and experimental designs will minimise welfare costs for animals when necessary for novel substances/cell types.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The design of experimental procedure will be interrogated against best practices guidance in order to ensure the most refined methodology is used, such as: local AWERB guidelines, those outlined on NC3Rs (e.g. ARRIVE guidelines), Guidance on Animal Testing and Research from the Home Office, LASA and RSPCA guidelines, PREPARE guidelines and published guidelines on tumour models.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly review publications (including those relating to the 3Rs) and attend conferences, discuss with other experienced groups, Named Animal Welfare Officer, hold

in vivo meetings to ensure that we constantly implement advances in techniques and non-animal alternatives. We also check experimental needs and designs with information on websites, such as Home office, the NC3Rs, LASA and RSPCAs.

97. The Neuroscience of circadian rhythms 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
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Circadian rhythms, Neurophysiology, Physiology, Chronotherapy, ageing

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how our brain generates and keeps daily timing in our physiology and behaviour, such as our sleep-wake cycle, mealtime, and peak concentration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 daily or circadian body clock is one of the most important timing systems in our body, ensuring that the activity throughout our nervous system and body is appropriately aligned with our physiology and behaviour across the day and at night. These demands include, for example, what time we sleep and wake up, our peak ability to process information, the best time to eat, and optimisation of brain toxin clearance. Disruption of these daily

rhythms can lead to severe health consequences, including premature ageing, increased risk of cancer and cardiovascular diseases, eating and mental health disorders.

In mammals, the master circadian clock is found in a brain region called the suprachiasmatic nucleus (SCN). Although the SCN is the master clock, circadian clocks are also found in many areas of the brain and body, which provide circadian timing at a tissue-specific level. For example, brain clocks aligning the activity of specific brain areas with the time-of-day at which these brain regions must be at peak performance, and heart muscle clocks increasing heart muscle tone during wakefulness and not during sleep. These extra-SCN circadian clocks form our extended circadian system (synonymous to our cardiovascular system, for example), and signal to one another through poorly understood mechanisms.

The overarching aim of the project is to understand how the SCN generates and coordinates circadian timing in the nervous system to organise bodily functions that are crucial to daily life, and how the different clock systems in the brain interact to maintain timing coordination in the circadian system across the day and at night. In doing so, we hope to unravel how and when disturbances and misalignment in the circadian clock network lead to ill-health and poor welfare, and how old-age and diseases, such as dementia, act to disrupt circadian timing in the body, leading to exacerbation of disease condition and further welfare degradation. Our ultimate goal is to use this new knowledge to help to generate appropriate pharmaceutical and non-pharmaceutical therapies to combat circadian rhythm disruption at the level of the body, when individuals in our societies are faced with modern life challenges, such as perpetual shift-work, travelling across time-zones and/or poor-health. Knowledge generated by this work programme, will also contribute in the long run towards our understanding of Chronomedicine, thus at what time-of-day should some medications be taken and for how long, and lighting conditions that patients in hospitals should be maintained under for rapid recovery. Indeed, this knowledge may also be used to improve the welfare of farm animals and animals used in research.

What outputs do you think you will see at the end of this project?

The immediate direct output will be from publication in high-profile international journals. My work has an excellent track record of producing such publications, and the intended programme of work under this PPL is a continuation of my research published in these high-profile Journals (such as Science, PNAS, PLoS Comp. Biol., J. Neurosci, eLife, etc, please see above).

Specifically from this programme of work, we expect to provide detailed information on how the novel “analogue” mode of electrical communication in the circadian system collaborates with its “digital” counterparts to generate and signal circadian rhythms in the body. This work will provide very rich and interrelated datasets at the molecular, cellular, brain circuit-wide, physiological and behavioural levels during health and youth. We will also determine how these multi-scale information are affected during ill-health and old age. This project will not be producing new animal models, but in the future we will be looking to cross our existing lines (such as our Per1-Venus reporter line) with other disease models (such as Alzheimer’s disease models) to provide useful tools and data for circadian biologists and other neuroscientists interested in contributing to our understanding of how clock rhythms are compromised during ill-health. We will seek further amendments to our PPL when the need to expand this to disease models emerges.

Who or what will benefit from these outputs, and how?

In the short term, the data generated by this work will initially be used by my group to shape our future research direction and interests. This work investigates some fundamental neuronal and glial signalling processes in the brain, and as such our publications (peer-reviewed journals and conference presentation) will influence researchers in the circadian and in the wider neuroscience communities. It is realistic to expect that this work will uncover new fundamental principles in circadian neuroscience and brain cell signalling pathways, exposing new avenues and possibilities for novel drug targets, while igniting testable hypotheses for drug repositioning. So, in the mid-to-long term, the pharma industries could therefore benefit from these findings. This work will generate rich in vitro and in vivo datasets which are likely to provide substantial opportunities for further computational analysis and modelling. The applicant has good relationships with a number of theoretical neuroscientists and mathematicians (in local, national and international institutions) and is committed to making data and mathematical codes freely available beyond the life of this project to any parties that can make further use of it. This in the long run may progress to the development of new research methods and technologies to facilitate work and data interpretation in the circadian field and neuroscience, which will ultimately lead to reduction and refinement of work with animals.

Part of our work program is dedicated to communicating our findings and understanding to the general public and patient's groupings, using these interactions to learn more about the human perspectives of circadian disruptions. The aim in the short-mid-to-long term is to impact and empower the patients by improving their understanding of the link between circadian disruption and mental disorders, for example, and how small changes in everyday routines and improved health management can make a big difference to their quality of life. This could have a good social impact leading to improved lifestyle choices on daily habits (e.g. feeding, bedtime), which in the long run could lead to improved healthcare policies.

How will you look to maximise the outputs of this work?

We have an excellent track record in collaborating with mathematicians and computational neuroscientists. Once we have published the data, all mathematical codes and analytical tools will be made freely available to the scientific communities.

We will continue to publish our work in peer-reviewed journals, and where possible involving press- releasing our work to maximise visibility.

We will continue with public engagement and disseminate our work at conferences.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult mice. The overall aim of the project is to understand how the brain

generates circadian rhythms in our physiology and behaviour. Mice will be used as the broad anatomy and physiology of this species is well understood and provide the best compromise in terms of correlating with human biology whilst exhibiting the lowest level of sentience. The mouse is also the animal model of choice for circadian biology research across the world. Therefore, we will be able to compare our results with the large body of literature available. In addition, many research and analytical tools have been developed for the mouse, making the data obtained more interpretable, reproducible, and refined.

Our aim is to understand how the brain organises circadian rhythms during youth and ageing. So, we need to use animals of different ages.

Typically, what will be done to an animal used in your project?

Typically, an animal will be born in the breeding room where it will be maintained until tissue collection. Some animals, however, will be placed in an altered light-dark cycle (circadian light/dark regimes) before tissue collection. During the altered light-dark cycle, a mouse will appropriately go through one or several light changes to prepare its circadian system before tissue collection. These light-dark cycles could be an advance of 12 hours and/or placed in constant darkness or received a pulse of light at a given time-of-day, for example, and may last from 4 days to 20 weeks, depending on the nature of the experiments. Training the circadian system in these various light/dark condition(s) is important as we study the effects of the light/dark cycle on our daily rhythm and how this affects sleep timing.

Sometimes, we will also measure the wheel-running activity of the mouse in a running wheel (as we would with our pet hamster at home) so that we have an understanding of the light/dark effects on behaviour. For collecting brain tissue from the mouse, typically, the mouse will be placed under non- recovery anaesthesia and then be killed by cervical dislocation.

What are the expected impacts and/or adverse effects for the animals during your project?

As these are all very mild procedures, we are not expecting adverse effects on the animals for most of the work we do. However, when we measure circadian rhythm using a running wheel, we would unavoidably have to singly-house the animal. Social isolation can be stressful, but the benefit is we will be continuously monitoring their circadian rhythm and sleeping patterns. We will therefore be able to monitor signs of distress. We will provide the animals with good environmental enrichment as much as possible to reduce stress. Fortunately, running wheels also reduce stress during social isolation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All our procedures are considered mild.

A small proportion of animals (< 1%) may be singly housed for up to 20 weeks. Of those, < 1% may experience some long-term mild harm.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are studying how circadian rhythms are generated and signalled in the brain and body of mammals and how these signals “break down” during environmental perturbation and disease with the view to relate and translate the findings to understanding the human circadian system. Due to the complexity of the nervous system and the intricate interactions of the circadian system in mammals very little information is available. As such, the only route to advancing our learning is through essential animal research work in an appropriate species. In addition, as incredibly advanced as our modern technologies are, they are not sophisticated enough for us to interrogate them on cell and circuit functionality as they exist in the whole organisms. Indeed, non-protected animals, such as worms and drosophila, do not possess brains that are complex enough to investigate our aims and will provide inappropriate information.

Which non-animal alternatives did you consider for use in this project?

We have considered using computational and mathematical modelling and non-protected species such the worms and flies.

Why were they not suitable?

However, as incredibly advanced as our modern technologies are, they are not sophisticated enough for us to interrogate them on cell and circuit functionality as they exist only in whole organisms. Non- protected species, such as worms and drosophila, do not possess brains that are complex enough to investigate our aims and will provide inappropriate information.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

We have estimated the number of animals required based on pilot studies and our published data. We have extensive experience in using these models and scientific methods to extract and analyse the data, and can therefore accurately estimate the number of animals we will need per group to obtain statistically valid results. As we generate more results, we will continue to assess group sizes 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. We are also using mathematical modelling to make appropriate predictions that can be tested in future work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have made use of all local support including our statistician and other resources such as the NC3Rs Experimental Design Assistant and the ARRIVE guidelines. We have also made use of our long-term collaboration with mathematical modellers to identify some of the meaningful targets to focus our investigation. We have also sought advice from collaborators at other research facilities when designing experiments. We are also using animals that are engineered (transgenic) to provide the most accurate results possible, thereby reducing statistical variance in our results. We are also using animal species that are models of choice in the circadian research field, thereby allowing us to refine our methods and optimally interpret our results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our use of long-term cell culture systems will reduce the number of experiments that need to use live animals. We conduct pilot experiments where necessary to ensure that our experimental systems are optimised before conducting full studies. Where possible we will carry out studies in a step-wise manner to minimise the number of animals used. These are appropriately advised by our mathematical models. Our collaborators work on different parts of the brain and body. So, we will share tissues with them to reduce the total number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using adult mice that contain genetic markers in their brain circadian clock cells. In these models, we will be able to monitor and manipulate circadian activity ex vivo after placing the animals in appropriate light-dark conditions. The animals will be placed from 4 days to up to 20 weeks in these light-dark conditions. We will then perform imaging and record electrical activity from their brain ex vivo.

Why can’t you use animals that are less sentient?

Our aim is to understand how the mammalian brain generate and perceive circadian time to organise physiology and behaviour across the day-night cycle. For most of the project, it is important that the animals possess a certain level of sentience, so that they can appropriately perceive the light-dark cycle to generate the complexities in the brain and physiological responses that are comparable to humans, rather than a basic reflex reaction.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At all stages of the project, we will conduct welfare assessments on the animals to evaluate the impact of procedures. We will accordingly review our procedures to determine whether aversive procedures can be minimised without adversely affecting scientific outcomes. We will also seek advice from the named people and other scientists working in the field.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will keep up to date with relevant scientific articles and attend seminars and conferences. We will also keep up to date with published guidelines by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) and Laboratory Animal Science Associations (LASA).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date by reading scientific articles and attending conferences. We will also liaise frequently with the named veterinary surgeon, named animal care and welfare officer, and our named National Centre for the Replacement, Reduction and Refinement on the use of animals in research (NC3Rs) support for expert advice and best practice. In addition, we have signed up for the newsletters by the National Centre for the NC3Rs, and we will regularly consult their website and attend webinars to keep our knowledge in this area up to date.

98. Epigenetic inheritance and the control of developmental and physiological processes

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

genes, gene regulation, developmental disorders, genetics, epigenetics

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged
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's the aim of this project?

We aim to understand the function and regulation of genes that are important for animal body function and development throughout the life-course.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 study has important implications for the health and well being of the individual and their offspring. Epigenetics refers to a range of factors that regulate the activity of genes, a controlled process that is important for ensuring that genes are expressed properly in the right cells at the right time.

When epigenetic processes do not work properly, cells behave abnormally leading to diseases such as growth abnormalities, disorders of the nervous system, metabolic diseases such as obesity and diabetes and cancer *. For example, Angelman syndrome,

an imprinting disorder leading to severe intellectual disability affects 1 in 12,000-1 in 20,000 people worldwide, and Prader-Willi syndrome, another imprinting disorder linked to intellectual disability, compulsive eating and obesity affects 1 in 10,000-1 in 25,000 people worldwide.
Epigenetic marks are set early in development and altered over time. While much can be learned about epigenetics from studying cells in a dish, information about the ways epigenetic changes are regulated can only be understood by investigating the whole body throughout its life.

* https://www.nature.com/scitable/content/epigenetic-diseases-and-their-causes-and-symptoms-37397/

What outputs do you think you will see at the end of this project?

We will generate new knowledge, to be shared with others, which will lead to the discovery of important factors that change gene expression without altering the genetic code in the form of DNA, this field is known as epigenetics. Specifically, processes in the contexts of the nervous system, growth and development and lifestyle-related diseases, for the benefit of humanity.

We will also understand fundamental processes associated with health and wellbeing in parents and the effect on offspring when these are perturbed genetically or environmentally. This has the potential to lead, in the long term, to new ways to detect diseases, predict their development and identify new ways to design medications for treatment.

Our findings will be published in scientific journals which are accessible to all, and our data will be placed in publicly available online resources. These published data will support future funding applications and has the potential to take the scientific discoveries to the clinic and the medical field.

Who or what will benefit from these outputs, and how?

During the 5 year course of the licence:

Our research in epigenetics will have an impact on our understanding of genetic diseases by identifying regions of the DNA where abnormal changes might lead to developmental and life-style related disorders, and cancer.

Our research on nutrition, before and after birth, will help understand more about why babies can be born with an abnormal weight. Additionally, our findings will look at the long-term impact nutrition has on their health and wellbeing over the lifecourse. This work will also have an impact on our understanding the benefits of breastfeeding for babies and mothers.

Our research on epigenetic processes being passed from parent to offspring, will help us understand whether and how environmental changes influence our DNA. We will investigate the extent of these environmental influences and how or if they can be inherited from parent to offspring and even to the next generations.

How will you look to maximise the outputs of this work?

Our research is highly collaborative and we will actively seek to contribute our expertise, technology and materials, positive and negative data to others and are often sought out by colleagues who wish to collaborate with us. We have a good track record in this, as evident from the publications that we co- author with others.

We will continue to establish and participate in national and international scientific organisations to maximise the impact of our research and add value altogether. We will continue to present our work prior to publication at conferences, seminars and lectures.

We actively collaborate with scientific companies to screen for the best tools to be used in the research community and weregularly report successful and unsuccessful results.

We are obliged by our funding agencies to publish our studies only in peer-reviewed open access journals.

Species and numbers of animals expected to be used

- Mice: 25,550
- Zebra fish (Danio rerio): 11,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and zebrafish are used to investigate aspects of our studies. An extensive catalogue of genes already exists for these organisms and they are widely used across the scientific community which allows for accurate comparisons between data. Also, well-established procedures already exist that are not harmful for the animals which can be used to mutate the genes or change their regulation. Many of the genes found in mice and zebrafish are found in humans too.

We study developmental processes therefore it is necessary to study all life stages.

Typically, what will be done to an animal used in your project?

For the vast majority of our work animals will be mated and material collected, post mortem (after death), at various life stages.

To study embryonic development, female mice will typically be injected into the abdomen, with a hormone to stimulate egg production, this is done twice in an animal's life and eggs are collected post mortem.

To assess metabolic processes animals may receive an altered diet, and substances such as glucose or insulin, will typically be administered once into the abdomen, followed by blood sampling of one drop, using a blood sugar monitor similar to that used by diabetics. Up to 6 blood samples will be taken from the tail vein over 4 hours. Typically, a mouse will experience each of these above procedures.

Some animals will undergo 1, 2 or sometimes 3 behavioural tests typically to test motor skills, anxiety and spatial navigation.
To evaluate mouse milk the mothers will be separated from the pups for up to 4 hours to allow milk to accumulate in the mammary glands. After this 4-hour period of separation, mothers will be anaesthetised and will have an injection of hormone to stimulate milk production. The nipple will be gently squeezed to release the milk. Mothers will then recover on a heated pad, and then returned to the pups that will remain in the nest undisturbed in a heated cabinet.

To study neurogenesis in zebrafish, animals will typically be singly housed, undergo behavioural tests, may have blood taken, and tissue sampling under anaesthesia such as fin clip. The fin clip is the collection of the very tip of the tail fin under brief anaesthesia. The fin tail grows back shortly afterwards. Taking a fin clip does not interfere with swimming.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals will undergo well-established procedures and will experience no adverse effects.

When new mouse lines are created or new crosses are bred we do not know the effects these will have on the offspring. Occasionally, these new lines may generate offspring with delayed developmental growth and the offspring may be born with a lower birth weight compared to their wild-type litter mates. Sometimes these mice with a lower birth weight regain a normal weight during adulthood.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: Sub-threshold - up to 60% Mild - up to 30% Moderate - up to 10%

Zebrafish: Sub-threshold - up to 60% Mild - up to 25% Moderate - up to 15%

What will happen to animals at the end of this project?

- 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?

We are performing experiments to assess the health of the whole organism. Our observations include multiple body systems and organs working together in a living animal, and multiple generations which we explore as part of our objectives. Such experiments

cannot be performed in a dish which usually only allows studying simple systems and very few cell types. Epigenetic states in a dish are very different to their natural state in the living animal, and currently are unable to recapitulate the physiological status.

We are studying mechanisms and pathways in a developing organism in embryonic and adult stages, therefore, we need to look at different time points throughout development. As cells in a dish are exact copies of each other this means that changes occurring in developing cells cannot be seen.

Which non-animal alternatives did you consider for use in this project?

We use non-animal alternatives as much as possible before moving on to animal models. Such alternatives include cells in a dish experiments, which allow us to answer simple questions without a live animal. We perform initial experiments in cells to test some of our hypotheses and choose the best candidate genes and pathways to focus on before moving on to animal experiments. This way, we can reduce the total number of animals we use.

In addition, we use cells to mimic as much as possible developmental processes in dishes. Such technologies make it possible to study early developmental processes, such as embryonic development.

To reduce animal work, we use publicly available sources and re-analyse existing data wherever possible with computer-based work and analysis.

Why were they not suitable?

Very often, the non-animal alternatives are not suitable. We cannot conduct experiments that address the health of an entire organism in the dish, which lacks the complexity and the interactions occurring in a physiological context.

Epigenetic states differ very significantly between cells in a dish and a living organism and to be able to conduct experiments that produce reliable and relevant data, we must use animal models.

When studying maternal-offspring interactions, there are no alternative non-animal methods that are sophisticated enough to address behavioural, hormonal, epigenetic and physiological questions which are very important aspects of our research programme.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 based on our work over the past 30 years. Key members of our team have enough knowledge and experience for their research field. We estimated the numbers depending on their projects using robust statistical analysis software on data generated from initial experiments using a small number of animals or cells in a dish (pilot studies) or from past experiments to allow us to determine our sample size.

Our research requires the use of large numbers of animals. The nature of our work focuses on the differences inherited from the mother and father. In order to see such differences, we need to follow the transmission from the mother and father in separate breeding pairs, which doubles our animal numbers for every experiment.

Additionally, in many settings of breeding pairs, we only need to study either males or females, for example when studying mammary gland biology, which means we only use 50% of the animals that are born.

Moreover, many experiments only focus on a certain mutation which is only carried by 25% of the animals that are born. For these cases when many animals that are born will not be used in such experiments, we have a sophisticated communication platform within the lab, to ensure maximal usage by other researchers, for example for breeding, pilot experiments, tissue collection.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use online tools such as NC3Rs EDA https://www.nc3rs.org.uk/experimental-design-assistant-eda and PREPARE guidelines https://norecopa.no/PREPARE, and NC3Rs website https://nc3rs.org.uk/3rs- advice-project-licence-applicants-reduction . Additionally, we consult collaborators or experts when we need to design new experiments in which we need to acquire additional expertise.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

efficient breeding

We work closely with animal technicians in the facility, who will advise us if breeding pairs are not producing any litters or producing fewer litters. Furthermore, all breeding information is recorded in our online colony management system, where we record all animals and their procedures. Therefore we will notice immediately if breeders have problems.

sharing of animals, tissue samples, and cell lines

We communicate regularly to share animals, tissue samples, and cell lines. We use shared files to summarise and share the information of stock tissue samples and cell lines. All animal information has been recorded in colony management system, therefore animal users can easily check which animals are available.

We share animals, tissues and cell lines with our collaborators worldwide. This means that others do not need to generate new mutants or samples, minimising animal numbers.

preservation of mouse strains

We freeze sperm or preimplantation embryos from mouse strains that are not planning to use at least for a couple of years. We have deposited some lines into The European Mouse Mutant Archive which are made available to the scientific community.

pilot studies

Where possible we conduct initial studies on cells in a dish or with a small number of animals to determine whether experiments are then required in larger numbers. Occasionally our scientific questions can be answered by laboratory based experiments alone.

We optimise protocols with small-scale experiments with wild-type samples before conducting experiments using mutants.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We work on mice as it is a well-established system to genetically manipulate this organism as well as to maintain controlled breeding. It is also possible to carry out phenotypic analysis of mice. Due to the genetically homozygous background of laboratory mice, genetic background effects do not affect analysis and make it easier to interpret results with fewer animals being required. The mouse genome has been well studied and characterised, therefore protocols for embryo and reproductive manipulations are well-established and safe for the mouse.

Many of our new mouse lines have been generated using CRISPR technology, which can generate multiple mutations using a single targeted event. This therefore requires the use of less mice and speeds up the generation of new mouse models. Mutations can then be selected for and an extensive breeding scheme can be avoided.

We also work on zebrafish which share a high degree of genetic and tissue similarities to mammals. As zebrafish lay as many as 200-300 eggs per mating, fewer animals are required per breeding programme. The eggs are laid outside the body therefore offspring can be studied whilst avoiding harm to the mother.

Why can’t you use animals that are less sentient?
Our research aims to assess mammalian gene function therefore the mouse is the best model for our work. Due to the lack of imprinting in species such as the zebrafish, we cannot use alternative species for most of our research. Our studies aim to look at the function of imprinted genes throughout development so immature life stages cannot be studied alone.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use well established protocols and will carry out pilot studies before starting procedures. Procedures will be discussed with experienced collaborators to minimise welfare costs for the animals. Any animal that has been through a procedure will

continuously be monitored at regular intervals examples include observing changes in locomotion (zebrafish), signs of gasping at the surface of water (zebrafish), sitting at the bottom of the tank (zebrafish), scoring sheets during procedural changes, weighing (mice), heat mats (mice) and providing post operative bedding (mice). When appropriate, pain management such as the use of medicated palatable substances for voluntary treatment, e.g. flavoured jelly, paste or milk shake liquid will be given to mice or appropriate pain-relief drugs will be dissolved in the water for zebrafish.

Mice are typically group housed with enrichments such as chew blocks, tunnels, mouse houses and nesting material. Zebrafish are typically group tanked and provided with enrichment if needed. We are always open to new suggestions by the NACWO for enrichments, such as running wheels etc.

If possible, for any new mouse lines, inducible constructs will be used so that mice only display a phenotype (visible physical characteristics such as behaviour or growth and appearance) once the deletion is induced. We will refine the humane end point for new mouse lines and will seek advice from the Vet and the Home Office Inspector if needed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our lab has been conducting embryological work over the last 30 years, therefore we have published protocols which ensure experiments are conducted in the most refined way. For any new procedures we will review published protocols from experienced collaborators before conducting any experiments.

We use ARRIVE guidelines to ensure our experiments are performed in the most refined way, and for the responsible reporting of results. This includes a thorough study design, sample size, inclusion and exclusion criteria, randomisation, blinding (where possible), outcome measures and state of the art statistical methods.

We use PREPARE guidelines which includes three areas that determine the quality of the preparation of animal studies:

Formulation of the study (literature, ethics, design etc)

Discussions between us and the animal facility (objectives, time-scale, facility evaluation, training and health assessment)

Methods (test substances, quarantine and monitoring, housing and husbandry, procedures and humane end point)

Specifically, for zebrafish we will ensure that our experiments are conducted in the most refined way, according to NC3Rs https://nc3rs.org.uk/zebrafish-welfare and enrichment provided if needed such as artificial plants as recommended by the Zebrafish Husbandry Association https://zhaonline.org/.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly discuss with the Named Persons at our institution on the best practices and new approaches in the 3Rs, including using the NC3Rs website pages https://nc3rs.org.uk/resource-hubs. We have subscribed to the NC3Rs e-newsletter to

receive information on the latest 3Rs developments as well as information on NC3Rs events and workshops, which we can attend. We consider the latest practical guidance from Laboratory Animal Science Association (LASA), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA). Additionally, we use external resources such as Jackson labs database https://resources.jax.org/. Lastly, we will maintain contact with our local NC3Rs Regional Programme Manager for advice on implementing advances in the 3Rs.

99. Stem cell regenerative medicine for neurodegeneration

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
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Stem cells, Therapy, Neurodegeneration, Behaviour, Neuropathology

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the project is to validate a novel pharmaceutical advance, a stem cell derived secretome, as a treatment for multiple neurodegenerative conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Dementia is a growing world-wide problem with recent data indicating that the prevalence of dementia will double in Europe and triple worldwide by 2050. Currently an estimated 1 million people are living with dementia in the UK alone. Dementia is an umbrella term describing a gradual and progressive loss of cognitive abilities cause by disease.

Alzheimer's is by far the most common form of dementia, while all common forms of dementia display specific brain changes in specialised brain areas. Other conditions, such a motor neurone disease and Parkinson's disease are also defined by similar changes in specific brain regions. This suggests an overlap in the molecular pathways that give rise to these conditions, and thereby suggest that potential therapeutics may also exploit these similarities. The specific brain changes that define these conditions all feature the aberrant accumulation of aggregates of proteins in the brain.

Currently Only 4 drugs, offering symptomatic relief, are licensed for the treatment of dementia (although none target the underlying pathology). Although treatments undergoing current clinical trials aim to reduce the predominant pathological feature of each of the different types of dementia, none of these targets all pathologies. Currently, no phase 3 trial has met its primary goals. Therefore, there is an urgent unmet clinical need to develop therapeutics that target all pathologies and halt/reverse clinical symptoms.

What outputs do you think you will see at the end of this project?

The programme of work is designed to determine the efficacy of a novel therapeutic approach to neurodegenerative disease related pathology and clinical symptoms. The expected benefits are two- fold: validation of the therapeutic potential for stem cell generated secretomes and a clear understanding of the link between pathology and clinical symptoms. Outputs generated will consist of a substantial body of published work detailing brain changes co-incident with behavioural deficits and responses to therapeutic treatment. This will provide a valuable resource that will aid research into treatment of Alzheimer’s disease and other neurodegenerative conditions.

Who or what will benefit from these outputs, and how?

The impacts of these outputs will help in the in the early stages of translational research into different neurodegenerative conditions, initial results will be reported after the first year of the project. Longer term benefits will be realised through translation of the key findings into disease modifying treatments. These treatments will specifically benefit patients with dementia, motor neurone disease and Parkinson's disease.

How will you look to maximise the outputs of this work?

Impact of outputs will be maximised through publication of results in open access scientific journals and through dissemination at international conferences. In order to maximise this, outputs will also be presented in lay terms to non-scientific audiences through articles in The Conversation, dissemination at Cafe Scientifique events and through Webinars. The project is a collaborative effort involving behavioural scientists, bioinformaticians and molecular biologists. Through their respective networks information and outputs will be disseminated.

Species and numbers of animals expected to be used

- Mice: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Four mouse models have been selected that model Alzheimer's disease (AD), Parkinson's disease (PD), Frontotemporal dementia (FTDP) and motor neurone disease (MND). These present with only a single brain pathology representative of the disease they are modelling. All models have well characterised temporal appearance of cognitive and/or motor and pathological phenotypes and will be investigated at an age coincident with already established pathology and behavioural deficits, this will be either adolescent or adult stage dependent on the model. Primary and secondary endpoints will be behavioural (mobility and/or cognition) and pathological (brain pathology). The choice of life stage has been determined by the known onset of pathology and behavioural changes.

Typically, what will be done to an animal used in your project?

Following acclimatisation and habituation, each animal will undergo behavioural testing of mobility and/or cognition for a few days to capture their baseline abilities. After this each animal will be treated either with a placebo or a test compound that we believe will slow the animals behavioural decline and remove the hallmarks of the disease from their brains. The vehicle or test compound will be injected into the tail vein of each animal to maximise successful delivery of compound. Blood samples will be taken after 2 weeks and a further 2 weeks later the animals will undergo behavioural testing again to determine the effect. Following this, animals will receive another tail vein injection of either vehicle or test compound to mitigate against systemic clearance of test compounds, and again blood samples will be taken after 2 weeks. After a further 2 weeks the animal will undergo further behavioural testing. Once the behavioural testing is complete the animals will be humanely killed, and their brains collected for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that as the animals age over the duration of the project (around 10 weeks in total) that there will be a gradual worsening of the animals' cognitive abilities and for some of the models a worsening of their motor co-ordination. The impact of the worsening cognitive abilities will be mild, while the impact of gradual motor deficits will not advance enough to cause distress. The behavioural test they will perform will not have any adverse effects. As with any systemic administration, there is the possibility of irritation around the injection site which may impact briefly.

Expected severity categories and the 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 likely/expected severity of procedural harms is moderate in all animals. It is expected that there will be momentary stress induced by the tail vein injection. For the behavioural analysis, there will likely be mild severity due to unfamiliarity of behavioral apparatus, this will be short term as the animals become habituated to the apparatus, this would be expected in 100% of the animals. Procedural harms are associated with the phenotype of all the animals. Cognitive deficits will be mild and sub-threshold of moderate severity, 50% of all animals will fall into this category, those that display motor deficits will experience moderate severity, 75% of animals will experience this. Of those animals which present with a motor phenotype there is the possibility that a number may present with an

accelerated phenotype which could be classed as severe. While this is unlikely it could present in 15% 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?

The tissue affected by Alzheimer's disease and other neurodegenerative conditions cannot be donated by living human beings, while post-mortem tissue is not suitable for the physiological assays involved. Non-animal alternatives are not developed enough for us to use as an alternative.

At the cellular and systems level, most of the proteins found in mouse brain share similar functions to the proteins found in the human brain. This means that the major intracellular signalling pathways are highly conserved from mouse to man, and the higher functions of the brain, specific to mammals, dependent on regulation of such proteins are also conserved. This, combined with the relatively short lifespan of the mouse, along with the tools available for genetic manipulation (such as the introduction of mutations that give rise to the pathologies evident in the brains of individuals with different neurodegenerative conditions), make it the most appropriate model of the complex neurological systems to be studied that also recapitulates the cognitive and behavioural symptoms that present with these conditions.

Which non-animal alternatives did you consider for use in this project?

A number of non-animal alternatives were considered for this project, including:

- Immortalised cell lines
- Inducible pluripotent stem cells
- Human post mortem tissue

Why were they not suitable?

The nearest substitute we have to animals is immortalised cell lines, however, while cell lines are a valuable model for many functional and biochemical assays, they do not develop axons and synapses and so are not morphologically representative of specialised cells such as neurones. In addition, no such immortal cell lines currently exist that replicate the intracellular accumulation of proteins similar to those that occur in the brains of individuals with different neurodegenerative conditions. Similarly with inducible pluripotent stems cells, these can be isolated from individuals with specific conditions and so can reflect a specific disease state, however they also do not form the complex morphological networks with other cell types found in the brain.

The tissue affected by individuals with Alzheimer's disease or any of the neurodegenerative conditions cannot be donated by living human beings, while post-

mortem tissue is not suitable for the physiological assays involved.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals for the duration of the projects is based on:

our preliminary observations and previous experience of performing both behavioural and biochemical analysis in order to minimise the number of animals needed to see meaningful differences between the groups, and two different approaches to determining the numbers of animals used in each experiment, making use of both the NC3R experimental design app and the G*Power 3.1 programme. Numbers estimated have also taken into consideration that some animals may need to be excluded as ‘non-responders’ in the behavioural tests.

If through the course of the experiments we find that we can gain significantly relevant information with fewer animals, the study will be changed accordingly.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animal studies were designed using the NC3R's Experimental Design Assistant (https://eda.nc3rs.org.uk/), an online tool for improving the design of animal experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

By combining behaviour and brain biochemical changes (by taking the brains form the animals following behavioural testing for post-mortem biochemical analysis) we have optimised the experimental design to gather the most possible data from each animal. In addition, all tissue from each animal will be banked as a resource to other investigators to reduce replication of studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Frontotemporal dementia Tau mouse strain, these mice display the neuropathological and behavioural phenotypes evident in patients with frontotemporal dementia. Specifically accumulation of aggregated tau protein.

Alzheimer's disease APP mouse strain, these mice display the neuropathological and behavioural phenotypes evident in patients with Alzheimer's disease. Specifically accumulation of extracellular deposits of aggregated amyloid.

Parkinson's disease Alpha-Syn mouse strain, these mice display the neuropathological and behavioural phenotypes evident in patients with Parkinson's disease. Specifically accumulation of intracellular deposits of alpha synuclein protein.

Motor neurone disease Q331K mouse strain, these mice display the neuropathological and behavioural phenotypes evident in patients with motor neurone disease. Specifically, accumulation of intracellular deposits of aggregated TDP-32 protein.

While most dementias display evidence of multiple pathologies, the models chosen only display a single pathology thereby minimising suffering and distress. All the models chosen present with a non- aggressive phenotype and a single pathology. The methods chosen to study are standard behavioural studies and will cause limited distress and no lasting harm to the animals. The method of application of the therapeutic was chosen as the least invasive method to deliver the therapeutic to the model.

Why can't you use animals that are less sentient?

Neurodegenerative diseases are largely age-associated as the pathology that develops in the brains that give rise tot he clinical symptoms takes many years to develop in humans. Similarly, animal models of these diseases also take time for the pathology to develop. The models we have chosen to study develop pathology slowly and so a more immature life stage would not have the pathology present to cause the behavioural symptoms. The proposed project aims to address both the pathology and behavioural symptoms of these conditions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures are of the minimum severity level needed to provide the required effects. All behavioural tests are mild and will not put the animals under an unnecessary stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will make use of the Laboratory Animal Science Association publications to refine my experiments, specifically the LASA Position paper: 3Rs : Replacement, Reduction, Refinement 9216). Guiding Principles on Behavioural Laboratory Animal Science. Avoiding Mortality in Animal Research and Testing.

I will be guided by the NC3Rs Resource Hubs, specifically Blood Sampling

I will make use of the ARRIVE Guidelines 2.0 as best practice for reporting the outcome of all experiments.

How will you stay informed about advances in the 3Rs, and implement these

advances effectively, during the project?

I will stay informed about advances in the 3Rs through the monthly NC3R newsletters. In addition, we have an active Biological Services Unit users' group of which I am a member and from where I can learn and share advance in the 3Rs

100. Studies to improve the welfare of pigs

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
- 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

Animal welfare, Pigs, Social behaviour, Nutrition

Animal types	Life stages
Pigs	juvenile, adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Commercial pig production can result in a variety of challenges to pig health and welfare. We use behavioural and physiological indicators to investigate the causes and consequences of these 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?

Animal welfare is high profile ethical concern of citizens and consumers, and various funders (including policy makers) seek evidence-based information on which to base decisions. Our research responds to these needs by providing scientific evidence to support changes to law or guidelines, or assurance scheme rules concerning management practices and housing systems.

What outputs do you think you will see at the end of this project?

Our research leads to: i) guidance on best practice for pig breeders, pig producers and veterinarians, or
ii) new technologies or techniques to be used on-farm iii) evidence base for policy makers and assurance schemes devising rules and recommendations for keeping pigs. Farmed pigs benefit through improved welfare, and this is seen as a benefit by citizens and consumers; pig producers may benefit through more efficient production with fewer loses (e.g. depressed weight gain from ill health or aggression).

Who or what will benefit from these outputs, and how?

Various actors in the pig supply chain will benefit, including breeders, farmers and veterinarians. Policy makers and assurance schemes will benefit from a greater understanding of best practice to improve pig welfare.

How will you look to maximise the outputs of this work?

We collaborate with industry partners (for example pig breeding companies, veterinary companies, animal feed suppliers) as part of the science we do. Findings are also published in refereed journals, and disseminated more widely to stakeholders through for example open days, events, agricultural shows, and articles for the specialist (pig industry) press (e.g. pig world) and websites (e.g. thepigsite.com)

Species and numbers of animals expected to be used

- Pigs: 4176

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 welfare problems we study require the whole living animal to be studied (e.g. to record behavioural responses), and non-animal alternatives are not available. We aim to inform on the welfare of pigs kept in commercial situations. We use pigs at the appropriate age (between birth and adult) for the specific scientific question being asked- studies of social development and behaviour include pigs between birth and ~4 months of age.

Typically, what will be done to an animal used in your project?

In social stress studies, pigs reared under commercial stocking density, and mixed with unfamiliar animals may experience stress and injury as a result of aggression.

For gut health studies, some animals may grow poorly or become unwell as a result of experimental diets.

For studies of needle-free vaccination, some pigs will experience the brief pain and subsequent discomfort of receiving a vaccination.

What are the expected impacts and/or adverse effects for the animals during your project?

For social behaviour studies, any severely bullied pigs will be removed from the pen and any injured pigs will be appropriately treated or euthanized on the advice of the NVS. During isolation for behavioural testing, some individuals may become stressed and continually try to escape- these pigs will have their test ended and returned to their home pen.

For gut health studies, pigs which grow poorly or become unwell (beyond mild and temporary diarrhoea) will be removed from the study for treatment or euthanized on the advice of the NVS.

For studies of needle-free vaccination, some pigs will experience the brief pain and subsequent discomfort of receiving a vaccination. We expect any impacts to be brief, and worse in needle-injected control 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)?

Studies of gut health and needle-free vaccination are expected to be mild.

A minority of animals in social behaviour studies may experience moderate stress following mixing into a new social group (this is a common practice in pig farming which our studies replicate in order to better understand their impact). As such these studies are classed as moderate. After mixing, some pigs will typically fight to establish a new heirarchy. This can be physically tiring and result in skin scratches. Some losing pigs may be bullied by pen mates. Groups usually settle down and integrate over a few days. Other pigs manage to go through these situations with little in the way of physical interactions.

What will happen to animals at the end of this project?

- Kept alive
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Studying pig behaviour, welfare and responses to supplementation requires that living pigs be used. Scientific literature from pigs and a variety of other mammals is used to ensure that experiments build on existing knowledge, have focussed hypotheses and that the work is genuinely novel (and not being unnecessarily repeated). Our research group also regularly undertakes and publishes surveys, reviews and systematic reviews of the literature to further this end. Also, our focus on behavioural studies, and on positive

behaviour means that we often carry out experiments that do not cause suffering so do not need to be regulated by ASPA. The findings of these studies complement and inform our regulated work. Nutrient supplementation studies are typically preceded by in vitro studies when supplements are being developed, so that only those most promising are employed in animal trials. In addition, cross- fertilisation of ideas between studies in poultry and pigs can refine hypotheses.

Which non-animal alternatives did you consider for use in this project?

None exist for experimental work. As stated above though, we make use of published literature reviews and meta-analyses, and carry these out ourselves, we also make use of information from other species where relevant. This minimises the need for new experimental work, and improves the focus and design of experiments which are necessary.

Why were they not suitable?

Animal welfare is an outcome of sentient animal minds, and our research is about pig welfare. As such we need to use living pigs as our research subjects. Also, our research involves behavioural phenomena such as social behaviour interactions between pigs. This requires actual living pigs to show these social interactions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A series of designed experiments have been planned for each of the four areas of work (protocols), and these total animal numbers emerged from those planned studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animal use was reduced by using efficient experimental designs incorporating factorial design, blocking and randomisation, and by minimising animal numbers by using power analysis based on previous similar studies where information is available. Our studies are reviewed by an independent statistician.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

None of these are relevant here.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use a number of refined methods developed over the years. For example, where possible: pigs are habituated to handling prior to behavioural testing, saliva sampling rather than blood is used, or pin- pricks of blood rather than a larger sample, and ear tissue collected for DNA as part of routine ear tagging for ID purposes. Pigs are closely monitored when negative outcomes are expected so that they can be treated as soon as possible.

Why can’t you use animals that are less sentient?

Our studies are intended to relate to the behaviour and welfare of pigs, as such pigs must be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our scientists and technical team are motivated to develop the most refined methods based on their own skills and experience. We have refined our methods over a number of years, including training animals to accept handling e.g. for weighing, refining the mix of drugs we use for sedation prior to euthansia, use of pin-prick blood sampling rather than jugular venipuncture, and the use of hair, saliva or faeces for cortisol sampling rather than blood.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Published on-line best practice guidelines prepared by NC3Rs will be followed. Peer reviewed papers have been consulted to ensure robust scientific use of the specific techniques described in the protocols.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are aware of the training materials offered by NC3Rs, some of which are directly relevant to our protocols (e.g. best practice in ear vein blood sampling in pigs). We have confirmed that our protocols adhere to the available guidance. Our protocols are reviewed at pre-determined intervals and, at these review points, we will ensure that they are updated to reflect the most recent refinements in NC3Rs guidance. Technical staff associated with the work will also be encouraged to engage with the resources available for continued professional development of technical staff provided by NC3Rs.

101. In vivo models of joint degeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

osteoarthritis, abnormal loading, inflammatory arthritis, knee joint, drug therapies

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to determine the mechanical and inflammatory factors that play a role in the maintenance, degeneration and regeneration of musculoskeletal tissues. Understanding these links will help identify new targets for therapy and help make decisions on surgery and rehabilitation to improve care of patients and animals with arthritis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 determining how these factors regulate responses to mechanical loading, interventions may resolve the degenerative processes associated with ageing and exercise. These objectives will also contribute to the understanding of how mechanical factors control and maintain the material and structural properties of musculoskeletal tissues. The data will be

used to advance the clinical management of high burden, high morbidity diseases such as osteoarthritis and rheumatoid arthritis through translation to clinical trials and inform on the management, rehabilitation and prevention of these debilitating conditions.

What outputs do you think you will see at the end of this project?

The outputs of this project will likely include:

the generation of novel data that will improve our understanding of the molecular mechanisms and the interplay of known risk factors i.e. altered mechanical loading, inflammation and genetic predisposition that underpin the development of osteoarthritis.

dissemination of the data in scientific publications, conference meetings and public engagement activities.

generation of pilot data to support the submission of grants to continue the research.

identification of new targets for therapy or re-purposing of existing drugs which have the potential to improve treatments for patients and animals with arthritis.

Who or what will benefit from these outputs, and how?

Our research focuses on an interdisciplinary ‘molecule to man’ approach to address major scientific questions in the arthritis field. As life expectancy and the incidence of obesity increases, the number of OA sufferers in the UK will continue to rise. There is no treatment for OA, except joint replacement surgery which has a limited lifespan and is associated with poor outcome in many patients (e.g. 20% of patients undergoing total knee replacement are unhappy with pain and function 6 months after surgery). It is known that abnormal weight bearing on joints causes diseases such as OA, whereas normal loading is essential to maintain a healthy skeleton. The forces associated with protective normal loading and destructive abnormal loading in humans, and the way in which these forces influence joint function, pain and inflammation are largely unknown. The mechanoresponsive pathways already identified in some of our projects directly regulate inflammation and therefore are also of considerable interest in the progression of inflammatory arthritis. Understanding the link between joint loading, inflammation and pain will identify new therapeutic targets and inform surgical intervention and rehabilitation strategies to improve care of patients and animals with joint degeneration.

Short-term impacts of the project i.e. within the 5 year period will include:

validation of existing mechanically-regulated therapeutic targets for bone and joint disease

development of new therapeutics and re-purposing of existing drugs to reduce arthritis progression *in vivo*.

Long-term impact i.e. beyond completion of the project, may provide an opportunity to:

Identify sufferers of arthritis earlier before joint damage is extensive thereby reducing the need for joint replacements

Devise interventions that alter loading patterns through diseased joints to slow disease progression

Develop drugs to abrogate harmful effects, and mimic beneficial effects of loading

Devise patient-specific treatments targeting pain and inflammation to delay requirement for joint replacement

How will you look to maximise the outputs of this work?

Project outputs will be maximised by:

ensuring the timely submission of research articles for publication in open-access journals to ensure a wide readership

attendance at international and national conferences to disseminate data findings

attendance at public engagement events to discuss use of animals in medical research

collaboration with other academic research groups with a shared interest in musculoskeletal mechanobiology and development of interventions for arthritis

collaboration with Pharma with a shared interest in 'drugging' molecular targets or potential therapeutics for human and veterinary purposes.

Species and numbers of animals expected to be used

- Mice: 1100
- Rats: 175

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents (rats and mice) will be used for these studies as their joint anatomy/physiology sufficiently resembles human. These model systems are well-defined with specific and rapid timeframes to enable therapeutic intervention at the point of injury or in the early inflammatory phase to be performed.
Skeletally mature (> 12 weeks of age) will be utilised for all protocols to ensure that the skeleton has fully developed and is representative of an adult.

Typically, what will be done to an animal used in your project?

Wildtype (mice and rats) and genetically altered animals (mice) will be acclimatised for one week to experience being handled, exposure to the environment and, if necessary, training in techniques that will be utilised as part of the measurement outcomes e.g. walking along an enclosed beam to measure gait and extent of weight-bearing on all four limbs. All four experimental protocols will involve administration of an anaesthetic via inhalation, with the possibility of an injection of painkiller whilst under anaesthetic. Depending on which protocol is used, animals may:

receive a non-surgical loading stimulus to their knee to either promote long-term degeneration of the joint over a 42 day period or to induce post-traumatic osteoarthritis over a 21 day period, or

have a surgical procedure performed which involves opening the joint and damaging the meniscus to induce joint instability over a 21 day period, or

be injected with an agent, typically six times over a 56 day period, which promotes a sustained inflammatory response in the joint to mimic inflammatory arthritis over a 91 day period.

Depending on the experiment, animals may receive an injection of a substance, typically a drug, directly into the joint whilst under anaesthetic which may occur during the procedure itself and/or at a defined point following initiation of arthritis. Following these procedures, animals will be allowed to recover, closely monitored for any immediate adverse effects and returned to their cage/cagemates for continued monitoring. Animals will undergo routine well-being measurements (daily during the 1st week and then every other day) including weighing, monitoring of lameness by two observers and measurement of knee swelling using fine callipers.

Other measurements that may be performed include assessing weight-bearing, pain and gait (walking along an enclosed beam, assessing weight distribution or recording pressure applied on each limb respectively), or whilst under anaesthetic the imaging of joints using scanners or withdrawal of fluids
e.g. blood and synovial fluid.

What are the expected impacts and/or adverse effects for the animals during your project?

Experimental protocols are well-established in our group, and therefore the likelihood of adverse effects are low. Furthermore, to reduce the likelihood of this occurring, we have stringent procedures in place to minimise risk/harm to the animals. The primary adverse effect is likely to be pain experienced by the animal after the specific procedure and once the arthritis has developed in the joint; therefore a painkiller will be administered at the time of the procedure and continuous monitoring of animal welfare will be conducted over the period of study. At the end of the study, or earlier if necessary, animals will be humanely killed and joint tissues processed to maximise the amount of information collected and analysed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

One of the loading experimental protocols is expected to be low severity with minimal discomfort to the animals and the remaining three experimental protocols are expected to have moderate severity as they could induce discomfort and/or distress to the animals. The proportion of animals that will be utilised in experiments classified as low severity is approximately 20% (mice) with the remaining 80% classified as moderate (mice and rats).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Due to the nature of the project, animal replacement is not feasible. We have developed explant and cell based models to investigate the mechanisms of joint destruction and protection in specific tissues and use these models wherever possible. However, experiments carried out on isolated cells and tissues alone are unable to recapitulate the biomechanical environment and the biological interactions between the surrounding joint musculoskeletal tissues, and hence the complexities of how the entire joint responds to mechanical load. There are currently no whole joint non-animal models of arthritis available. Furthermore, validation of targeted interventions as therapies for arthritis treatment can only be truly assessed in *in vivo* models where we can directly measure how well they rescue the disease symptoms in the different joint compartments. This can only be addressed by conducting the proposed *in vivo* experiments.

Which non-animal alternatives did you consider for use in this project?

As indicated above, where feasible we have developed and utilised alternative *in vitro* approaches to animal studies. This has included the utilisation of material derived as a by-product of the meat industry (obtained from abattoirs) as well as the development of models involving isolated cells and tissues.

We are actively engaged in developing more complex 3 dimensional and multi-cell type models to increase the tools available to us, and other researchers, for *in vitro* studies. Such models are currently, and will continue to be, used where possible to elucidate cell specific mechanisms.

Why were they not suitable?

Due to the nature of the project, animal replacement is not feasible. An *in vivo* model system is required to recapitulate the biomechanical environment and the biological interactions between the surrounding joint musculoskeletal tissues, and hence the complexities of how the entire joint responds to mechanical load. The additional influences of interactions between the different tissues involved in joint loading, e.g. cartilage, bone, muscle, ligaments as well as the systemic effects, and how well drug treatments might rescue the disease symptoms can only be assessed by undertaking the proposed *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?

These numbers have been estimated based on our experience and animal usage over the last 5 years under my previous PPL. Using this information, we have considered the number of animals required to further investigate a small number of specific molecular targets as we refine the course of our research plan moving forwards. A typical pilot experiment for Protocol 3, for one target, will involve a maximum of 120 animals to account for dosing regime, plus or minus load as well as the drug intervention and six time points. Numbers per experiment will then be less once we have identified optimal dosing regime. Lower animal numbers have been estimated for the remaining protocols because they will be used to validate the chosen therapy in the different models of arthritis that they represent.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3Rs EDA was utilised to design the specific experiment in conjunction with the implementation of power calculations to ensure the minimum number of animals were used in the previous PPL. These have and will form the basis of our future pilot studies which will be refined, where needed, to inform on animal numbers required to investigate a defined number of identified molecular targets within this current PPL.

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 (using appropriate power calculations) will be performed for any new studies to minimise animal number use, and in addition to this, where possible, we will utilise 'control' data from our previous experiments (under a previous PPL) to reduce the number of untreated animals required i.e. animals which have not gone through the protocol. Furthermore, we will ensure the sharing of tissue, either by harvesting other joint components or organs at the endpoint or providing histological sections of the joint for analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We intend to use four different models (Protocols 2 - 5), all of which induce an arthritic disease in both wildtype and genetically altered animals (Protocol 1) including:

A well-defined non-invasive loading model in living mice and rats that will apply mechanical loading to long bones and joints of one leg under general anaesthesia while the other leg acts as a control. The model will either recapitulate a loading regimen that mimics long-term degeneration of the cartilage, akin to that observed in human primary

'wear & tear' OA (Protocol 2), or it will elicit load-induced rupture of the anterior cruciate ligament, akin to that experienced in human post-traumatic OA after a sports injury/accident (Protocol 3).

A well-defined surgical model in living rats that, under anaesthesia, surgically destabilises the meniscus (a key component in the joint that protects the articular cartilage from excessive loads), akin to that experienced in human post-traumatic OA after a sports injury/accident (Protocol 4).

A well-defined inflammatory model of arthritis in which this disease is induced in rats and mice, akin to that observed in OA and RA pathology (Protocol 5).

Each of these in vivo model systems will allow us to determine how the pain and tissue degeneration is instigated in arthritis, thereby providing vital information to further our studies into developing therapies to treat arthritis in both humans and animals. Two of the protocols are classified as mild severity (protocols 1 and 2) with minimal discomfort to the animals and the remaining three protocols (protocols 3-5) are classified as moderate severity as they could induce discomfort and/or distress to the animals. The primary adverse effect is likely to be pain experienced by the animal after the specific procedure and once the arthritis has developed in the joint; therefore a painkiller will be administered at the time of the procedure and continuous monitoring of animal welfare will be conducted over the period of study to ensure animals do not experience unnecessary pain, suffering, distress or lasting harm.

Why can't you use animals that are less sentient?

Due to the nature of the project, animal replacement is not feasible, although we are developing explant and cell based models to investigate the mechanisms of joint destruction and protection in specific tissues and use these models wherever possible.

Experiments need to recapitulate the biomechanical environment i.e. animals need to be weight- bearing in a similar manner to humans, to mimic the complexities of how the entire joint responds to mechanical load. This can only be addressed by conducting the proposed *in vivo* experiments in rodents, which are considered the least sentient model in this research area. Also, animals need to be skeletally mature to be able to recapitulate how the disease manifests in human adults, thus more immature life stages would not address the key research questions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Measures are in place to minimise the welfare costs for the animals which include:

acclimatisation of all animals for at least one week prior to the initiation of protocols such that they become familiar with handling, surrounding environment, cagemates and measurement methods.

monitoring of animals on a daily basis after the induction of arthritis to ensure no adverse effects on their health and well-being e.g. weight loss, appearance and lameness.

provision of appropriate soft bedding and nesting material to cushion affected limbs and maintain their body temperature during this period to minimise unnecessary stress and/or discomfort.

provision of gel supplements in the cage to ensure animals with compromised mobility have appropriate access to food.

provision of additional cage items e.g. tunnels to provide refuge for those animals with compromised mobility.

provision of painkillers, where appropriate, and swift implementation of humane endpoints to minimise distress or suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In line with our previous PPL studies, we will ensure that all experiments conform to best practice by following the ARRIVE 2 guidelines (which our previous relevant publications adhere to), in conjunction with advice and guidelines disseminated by the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will remain informed about 3Rs advances through our continued email 'sign-up' to the NC3Rs newsletter where good practice and regulatory updates are disseminated. We will also continue to attend the NC3Rs regional events and liaise with our regional NC3Rs manager to ensure effective implementation of advances. The PPL applicant currently sits on the NC3Rs Training Fellowship panel (2020 - 2023) and benefits from updates on good practice through this forum.

102. Reproduction Safety Tests on Agriculture and Industrial Chemicals

Project duration

5 years 0 months

Project purpose

- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Toxicology, Reproduction, Safety

Animal types	Life stages
Rats	juvenile, adult, pregnant
Mice	juvenile, adult, pregnant
Rabbits	adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project licence is to establish toxicological and safety data in animals following exposure to industrial and/or agricultural chemicals and veterinary products that Man may be exposed to. The studies performed will be designed to reveal any effects on mammalian development and reproduction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chemicals play an important role in daily life. Therefore, their safety for Man, other animals and the environment has to be considered carefully. By establishing sufficient toxicological and other safety data in animals, safe handling precautions may be determined thus protecting the health and welfare of hundreds (e.g. for a site limited industrial chemical

intermediate with limited potential for human exposure) to millions (e.g. industrial or agricultural product with world-wide market) of humans and animal species which may contact the materials concerned and facilitate the world-wide marketing and safe use of products. The projects performed under this licence provide safety data to facilitate sound regulatory decisions worldwide that protect the public and the environment from possible hazards. The regulated products have the potential to improve and enhance the health, well-being and quality of life of people and animals. For example, improved crop-protection increases food security, while development of safer chemicals and chemicals with reduced environmental impact is clearly beneficial for human and animal health and in environmental protection.

The projects undertaken in this licence use methodologies that are well established and known to produce accurate and reliable results that can be used in regulatory risk assessment. Furthermore, the studies can rapidly identify any overt toxicity which would cease the development of the test item and therefore enable the Sponsor to make a decision at the earliest opportunity to cease production: reducing the risk of possible human exposure and avoiding unnecessary expenditure and use of resources. The work performed under this licence will be undertaken in a GLP compliant laboratory thereby ensuring data integrity and accuracy.

What outputs do you think you will see at the end of this project?

Chemicals play an important role in daily life. Therefore, their safety for Man, other animals and the environment has to be considered carefully. By establishing sufficient toxicological and other safety data in animals, safe handling precautions may be determined thus protecting the health and welfare of hundreds (e.g. for a site limited industrial chemical intermediate with limited potential for human exposure) to millions (e.g. industrial or agricultural product with world-wide market) of humans and animal species which may contact the materials concerned and facilitate the world-wide marketing and safe use of products.

The outputs of this project will support the development and continued use of safe chemicals that do not impact on the reproductive health or well-being of humans. This includes data to inform of the potential risks on development and reproduction that may be induced by either direct or indirect exposure to the chemical.

The expected output from this project will be robust data, in the form of study reports, to enable the safety assessment. Study reports will be included in regulatory submissions to allow regulatory authorities (e.g. OECD, EPA, ECHA) to make judgements on whether to permit the licence of a chemical.

Who or what will benefit from these outputs, and how?

The projects performed under this licence provide safety data to facilitate sound regulatory decisions worldwide that protect the public and the environment from possible hazards. Regulated products have the potential to improve and enhance the health, well-being and quality of life of people and animals.
For example, improved crop-protection increases food security, while development of safer chemicals and chemicals with reduced environmental impact is clearly beneficial for human and animal health and in environmental protection.
How will you look to maximise the outputs of this work?

While much of the substance specific data generated is covered by confidentiality agreements, work on novel biomarkers, refinements in methodologies, protocols and techniques that permit a reduction in the number of animals required for specific study designs or to achieve specific end points are freely shared and discussed at Scientific conferences and other forums (e.g. attendance at regular NC3R meetings).

Although most studies will require the use of concurrent Controls to provide contemporaneous data for direct comparisons (to represent animals undergoing the same regulated procedures, administered the same vehicle, etc), data generated from Control animals is held in reference databases to provide information relating to normal biological variation, thereby enhancing interpretation of study findings.

Species and numbers of animals expected to be used

- Mice: 12,000
- Rats: 17,000
- Rabbits: 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.

It is generally accepted that the way in which a material is metabolised and distributed within a living body has a significant effect on how it works and its potential toxicity. Unfortunately, at this time, effects on complex interacting biological systems cannot yet be replicated using *in-vitro* or *ex-vivo* methods (non-animal models). Consequently, the use of animals is still an essential part of safety testing.

All studies performed under this licence will use the least sentient species practicable for achieving the study objectives. The majority of cases will involve the use of rats and mice as required by the regulatory guidelines. In embryo-foetal development studies only, a second mammalian species is required, with the rabbit typically selected and in terms of practicality and availability the rabbit is generally accepted as the preferred non-rodent species, as it is known to be sensitive to a range of toxicants.

Studies conducted under this Licence will allow exposure to mature adults and all stages of development from conception to sexual maturity to inform of potential effects on development and reproductive health.

Typically, what will be done to an animal used in your project?

The studies performed under this licence will use either pregnant or non-pregnant animals. Non pregnant animals are typically used on preliminary studies designed to assess the tolerance of the test material or to establish suitable dose levels for use on main studies which generally assess effects on the foetuses following exposure via the placenta. Animals will be acquired from designated suppliers. In many cases the supplier will provide animals which have been mated at specific timepoints so that the period of gestation is known. In pregnant rats and rabbits dosing will typically start on day 6 of the animal

gestation and continue up to the day before necropsy. Such studies will typically be terminated on day 20 in the rat and day 28 in the rabbit.

All animals will be dosed with the test chemical via a route that would mimic accidental exposure in man, typically in food, water or contact with skin at normally three dose levels and monitored closely for signs of toxicity.

During the course of the study the animals will be well cared for and will be closely monitored for reactions to treatment. Blood and/or urine samples may be collected in order to assess for treatment related effects and/or exposure to the test substance. Other end points will also be included as required to address specific concerns (e.g. functional observation tests).

At the end of the study the animals will typically be euthanised and a necropsy undertaken. This is essential because it is important to establish if the internal organs and or foetuses have been affected in any way and this can only be achieved by examinations by qualified personnel (Foetal Morphologist/Pathologist).

What are the expected impacts and/or adverse effects for the animals during your project?

It is anticipated that some animals will lose weight, or at least fail to gain weight at a rate consistent with normal weight gain. This may be attributed to a reduction in food consumption (also a potential adverse effect) but may be present even with normal food consumption.

Animals will be closely monitored for signs of discomfort and particularly signs of pain. Any animal showing such signs will be closely monitored and will be inspected by a Vet if considered necessary. No animal will be allowed to endure pain for long periods and remedial action which may include euthanasia will always be taken.

Where pregnant animals are used it is anticipated that foetuses exposed to the test material via the placenta may experience clinical reaction that may affect their well-being. Pregnant animals will be carefully monitored throughout gestation for signs that could indicate that pregnancy is being affected: for example, signs of distress or abortion.

Some study designs require assessments on the development and behaviour of the animals. Assessments include the monitoring of activity and behaviour and testing of an animals grip strength, these tests cause minimal discomfort only. All procedures performed on the animals are fully validated and established within the industry to cause the minimum distress and will only be undertaken by trained staff.

On completion of each study the animals will be humanely killed and a post-mortem performed in order to establish effects on organs and tissues which aid the evaluation of the toxicity of the chemicals.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that the majority of animals (circa 65%) will experience no more than Mild discomfort e.g. a small effect on clinical signs, body weight and/or food intake. Other effects are possible, but will be transient and will also fall into the category of Mild severity.

It is feasible that a further 30% of animals will experience effects considered to be of Moderate severity e.g. a more significant effect on weight loss and/or food consumption, as well as other effects such as lethargy and ptosis (half closed eyes).

In rare cases, certainly in less than 5% of all animals used, a more severe reaction to treatment may be experienced. This is typically due to unexpected toxicity in particularly susceptible animals. Animals reaching severe severity will be euthanised without delay in order to prevent further suffering.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is generally accepted that the way in which a material is metabolised and distributed within a living body has a significant effect on how it works and its potential toxicity. In addition, effects on complex interacting biological systems cannot yet be replicated in *in-vitro* or *ex-vivo* tests. Consequently, for the majority of chemicals it is imperative they are tested on living animals in order to assess for toxicity to tissue, organs and systems e.g. the cardiovascular, respiratory and reproductive systems following repeated exposure. It's particularly vital that studies to assess the potential for reproductive and development toxicity are conducted in animals at this time due to the wide range of dynamic and interacting processes that take place throughout the life-cycle.

Which non-animal alternatives did you consider for use in this project?

Sponsors will typically utilise non-animal alternative assays as part of the development programme for a substance (e.g. *in silico* modelling and *in vitro* metabolism studies). Such assessments would not be conducted under this project, however, where available, these data would be used to guide the selection of the most appropriate species and to confirm the *in vivo* study design (e.g. to confirm the species which show similar patterns of metabolism to those expected in man).

Whilst information from these alternative assays will contribute to the overall safety assessment, they cannot replace the need for *in vivo* studies at this time.

Why were they not suitable?
The use of alternative methods, including the use of dead animals cannot, at this time generate relevant data which supports the submission of safety data to international regulators; this is particularly so for studies into the effects on pregnancy and development whereby there are a vast range of molecular processes that interact and could be affected. The fundamental aspects of the safety data we require involves assessing physiological,

behavioural and biochemical effects following the administration of a test substance and this can only be achieved by using live animals.

Alternative methods such as *in-vitro* techniques will be used as much as practicable to supplement the work involving protected 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 establishment maintains detailed records pertaining to the numbers of animals used on projects each year, as well as the number of study types undertaken. By analysing annual trends and having knowledge of industry requirements, it is possible to project the number of study types we will undertake during the life of this licence, thus enabling the estimation of the number of animals required. In addition, a new building will be in use which has increased the capacity for rodent work by approximately 30 %.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The minimum number of animals will be used, recognising the fact that reduction is not achieved by using too few animals to achieve the objectives of the study. For Regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise, reference is made to internal guidance on study designs to provide the optimum number, balancing the need to achieve study objectives while avoiding excessive animal use. Project specific variations are used as required. The core study designs have been used extensively under the previous project licence and in other facilities and we have a track record of successful submissions and ability to eliminate unsuitable compounds. They are generally in line with those used throughout the industry.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Statistical input is sought, where appropriate, to strengthen the overall scientific quality and relevance of the studies to be performed, with power-sample size calculations performed for specific studies if necessary to determine group size. For preliminary studies, small groups are acceptable because of the potential use of overt toxicological endpoints. Where group sizes are sufficient data from definitive toxicity studies are analysed statistically.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 studies conducted under this project will typically use rats and rabbits, with mice also included where considered biologically relevant; these species are specifically requested by regulatory authorities due to their ability to identify possible reproductive and developmental toxicants, their good fertility, as well as due to the large amount of background data that has been accumulated.

It is generally desirable to use the same species and strain as in other toxicological studies. Reasons for using rats as the predominant rodent species are practicality, comparability with other results obtained in this species and the large amount of background knowledge accumulated. In the majority of cases outbred rodents and the New Zealand White Rabbit will be used as these strains have good fertility and provide sufficient foetuses/litter sizes for the assessment of test item-related findings.

In embryo-foetal toxicity studies only, a second mammalian species traditionally has been required; the rabbit being the preferred choice as a non-rodent species. Reasons for using rabbits in embryo-foetal toxicity studies include the extensive background knowledge that has accumulated, as well as availability and practicality. Where the rabbit is unsuitable, for example they do not show exposure, a second rodent species, for example the mouse may be acceptable and should be considered on a case by case basis.

Regulatory authorities require characterisation of toxicity at the Maximum Tolerated Dose (MTD) to ensure robust evaluation of safety before potential human exposure; it is, therefore, necessary to perform toxicity studies at high doses that produce overt toxicity, usually in terms of clinical signs or body weight loss or depression of weight gain against age-matched controls. Response to observed effects will depend on the objective of the study; on preliminary studies where the objective is to determine the MTD then doses will be increased until effects are evident; once clinical signs or extent of weight loss indicates that a dose is unsuitable for use on a definitive study then action would be taken to alleviate the clinical signs, usually this would involve termination of the sex/group or may involve reduction of the dose.

Definitive studies should also show evidence of toxicity and typically clinical signs or reduced body weight gain would be seen in the high dose animals following dosing: some clinical signs may be present for up to 8 hours after dosing but would be expected to show clear signs of recovery at this time. Generally clinical signs would be expected to be absent prior to dosing on the subsequent day although some animals may still show some moderate clinical signs. Clinical signs will generally be expected to be up to moderate in severity. However, because toxicity can become worse with increased duration of dosing or the exposure of animals to the test substance may increase over time, sometimes high dose animals and occasionally lower dose animals may show significant adverse effects and in this case action will be taken to alleviate the clinical signs such as withdrawal of the animal from dose, reducing the dose (if appropriate) or termination. In some cases, effects seen on definitive studies can differ from those seen on preliminary studies for no apparent reason. A single clinical sign is generally unlikely to be sufficient to warrant termination unless the presentation is severe and the combined effects of multiple signs will always be assessed for cumulative harm to the animal.

Preliminary studies when performed will allow the delimitation of appropriate intervention and humane end points. Definitive studies will be performed within a moderate severity limit, as the use of humane endpoints, careful monitoring and rapid response to observed effects will negate the need for a severe limit.

Where little is known about a substance, or a class of substances, before commencing preliminary studies consideration will be given to using staggered starts where the effects in one group are assessed before commencement of dosing groups at higher doses. In all cases, dose selection will be based on all available information, data from preliminary studies or in some cases, and data from published literature.

The methods (procedures) used will be validated, well established and commonly used within the research community. The administration of test substance, removal of blood, collection of urine for example will cause no more than transient discomfort or distress. Any signs of distress will be carefully monitored including the onset and severity of treatment related effects. Appropriate and swift action will be taken to avoid any undue pain or distress.

Why can’t you use animals that are less sentient?

The studies performed under the authority of this licence are primarily to assess the toxicity of materials when exposed to foetuses, as well as the parental animals. The use of immature life stages or species that are less sentient than these would not allow us to achieve the objectives of our studies.

Similarly, the duration of the studies which, in the case of multi-generation studies may be several months and studies often requiring behavioural assessments do not allow them to be performed under general anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures required will be undertaken by competent staff, each having undergone extensive training. Where necessary, current, industry accepted techniques for dosing and blood sampling for example, will continue to be refined under separate authority i.e. under another project licence, thus ensuring existing methods remain the most appropriate for minimising pain, suffering and distress to the animals.

Animals will be monitored immediately after undergoing a procedure for any signs of adverse effects and will continue to be monitored at appropriate intervals until it is deemed that further observations are not required.

Animals will be habituated to procedures whenever considered necessary i.e. when it is deemed that by habituating animals to a procedure distress will be reduced. Similarly, where appropriate animals may be trained to perform certain tasks thereby, minimising distress by removing the need to restrain an animal or involve direct contact during its performance.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The volumes administered to the animals and the volumes of blood taken will be in compliance with industry accepted guidelines. The primary guideline used is:

"A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes"; Karl-Heinz Diehl, Journal of Applied Toxicology J.Appl. Toxicol. 21 15-23 (2001).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Staff maintain a proactive attitude towards the 3R's. Several members of staff are already participants in Industry Forums which discuss the 3R's in some detail and report any advancements to relevant persons. These advancements will be discussed further and implemented into our standard practices, where appropriate. The company is an established leader in the development and application of the 3Rs in toxicology studies.

103. Disease-modifying therapy for amyloidosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

amyloidosis, monoclonal antibodies, immunotherapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop effective treatments for amyloidosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiac amyloidosis is a progressive and ultimately fatal disease that is now known to be much more common than previously thought. Promising treatments are in clinical development, but their mode of action is to inhibit amyloid deposition and disease progression. They are not expected to reverse organ damage, which is usually very advanced at the time of diagnosis. The treatments we will work to develop have the potential to remove existing amyloid deposits and restore organ function, thereby preserving and improving quality of life.

What outputs do you think you will see at the end of this project?

We expect to have identified monoclonal antibodies and immunotherapy treatments which can elicit amyloid clearance, as candidate medicines for the treatment of human amyloidosis.
We expect to have evaluated the potential of anti-amyloid antibodies for diagnosis and monitoring of amyloidosis.

We expect to have applied for one or more patents.

We expect to publish our findings in the scientific literature.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries will be the pharmaceutical industry, the academic community and, through engagement activities, amyloidosis patients.

The intended long-term beneficiaries of these outputs will be amyloidosis patients and their families, the pharmaceutical industry and healthcare providers.

How will you look to maximise the outputs of this work?

Patent protection will be sought, as appropriate, to maximise the likelihood of successful development of therapies and imaging reagents.

Commercial collaboration(s) will be established for clinical development of treatments and diagnostic reagents. Findings will be made available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used because it is possible to model human amyloidosis accurately, and because of the wealth of knowledge, genetic resources and reagents that enable successful use of mice. Adult mice will be used by necessity because the disease takes months to develop in mice.

Typically, what will be done to an animal used in your project?

Genetically altered mice will be bred and adults will typically receive an injection, into a vein. After allowing time for accumulation of amyloid (typically one month to six months), antibodies will be injected into the abdomen (typically twice or three times) to elicit resorption of the amyloid, and the mice will be killed (typically within one month of antibody injection) for evaluation of the response.

Some mice will receive larger numbers of antibody injections. Small blood samples (up to six) will be collected from some mice.

What are the expected impacts and/or adverse effects for the animals during your project?

The genetically altered mice are, on average, smaller than their wild-type littermates; this difference is benign. Most of the mice used for experiments will have amyloid deposits, but in smaller amounts than are typical for symptomatic clinical amyloidosis. For most animals, the impacts are expected (at worst) to be the minor and transient discomfort caused by injections and blood sampling. A minority of the mice used will be subject to repeated injections and/or 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 animal type)?

Sub-threshold: 65%; Mild: 15%; Moderate: 20%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The development, evolution and resolution of pathology causing clinical diseases in living organisms are extremely complex. Although increasingly sophisticated in vitro and in silico models are being developed, none individually or in combination are capable of adequately modelling the enormous complexity of the in vivo situation.

Which non-animal alternatives did you consider for use in this project?

Our overall plan of work includes appropriate in vitro studies, but for the questions we will address in this project, there are no suitable in vitro or in silico alternatives. There is no alternative to use of living animals to achieve the level of knowledge and understanding of these processes which is necessary for the new approaches to treatment which we are developing to be taken into clinical testing in humans.

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?

We will use only the standard experimental protocols with which we have extensive experience that we have established to provide robust, statistically rigorous results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experimental protocols are based on our decades of experience with relevant experimental models, which will guide experimental design so that the minimum appropriate numbers of animals are used. Experimental design will be informed by power calculations, to ensure statistically and biologically significant results.

We have lately developed a new highly reproducible animal model which resembles the human disease much more closely than previous models. These features reduce the numbers of animals required to give robust and valid outcomes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will manage our colonies of genetically altered mice to match as closely as possible the requirements for experiments, while ensuring the security of the colonies.

Where compatible with the primary purposes of experiments, additional samples will be collected for use in other studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project we will use a newly developed transgenic mouse model of cardiac amyloidosis closely resembling the human disease. We will only use simple non-surgical techniques, thus greatly reducing potential pain, suffering, distress or lasting harm to the animals.

Why can’t you use animals that are less sentient?

The newly developed mouse cardiac amyloidosis model is the only one that exist in mammals. There are no relevant models of amyloidosis in other types of animals. In order to achieve our goal of modelling the treatment of the human disease with new drugs, there is currently no suitable alternative to the use of live mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The welfare costs will be taken into account at the experimental design stage. The least harmful procedures appropriate will be used. Monitoring for any adverse effects, including pain, will be the highest priority, although the procedures do not cause more than minimal discomfort, for which pain relief medication has not previously been required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The guidelines issued by LASA and NC3Rs will followed as closely as possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult NC3Rs resources (website, publications, etc.), engage with our local NC3Rs Regional Programme Manager, maintain a dialogue with the NACWO and other staff who support animal research within the University, and we will stay up to date with the literature in the amyloidosis field. Advances in the 3Rs that may be relevant to the present programme will be implemented if they do not interfere with validity of experiments.

104. Drug and immunity 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
  - 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

leishmaniasis, cancer, drug, vaccine, phototherapy

Animal types	Life stages
Mice	adult
Rats	adult
Hamsters (Syrian) (Mesocricetus auratus)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project is to identify novel treatments for leishmaniasis and cancer, develop novel delivery systems to improve drug, targeting with the focus being on non-invasive routes of administration, and develop vaccine candidates for leishmaniasis. These two models allow the effect of changing the drug/vaccine formulation on delivery in different sites in the body to be studied.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many diseases or conditions require multiple treatments using a variety of drugs as the condition does not respond well to the clinically used drug/drugs. One way to improve treatment is to target the drug to the site of infection/condition so that a higher drug dose can be used locally. Targeting can be achieved by using a drug delivery system to deliver a drug to the local area, and minimising any adverse effects on healthy cells/tissues. In this project different conditions, namely leishmaniasis and cancer, are used as they are clinically important and they occur at different sites in the body. This allows targeting of drugs to different areas of the body to be studied and extends the findings of this project to other conditions. Leishmaniasis is an important neglected disease which is caused by infection with the protozoan parasite, *Leishmania*. It is transmitted by the sandfly vector and the World Health Organisation estimates that there are more than 1 billion people living in areas endemic for leishmaniasis are at risk of infection. An estimated 30,000 new cases of visceral leishmaniasis and more than 1 million new cases of cutaneous leishmaniasis occur each year. Visceral leishmaniasis is associated with an enlargement of the spleen and liver and it often associated with fever, weight loss and anaemia. It is fatal if untreated. Cutaneous leishmaniasis results in skin lesions which can be single or multiple and often occur at the site where the sandfly feeds. These lesions can become infected with bacteria and cause dry or wet sores. The lesions are generally not life threatening but they can cause scarring and disfigurement which can cause social stigmatism and affect a person's mental health and well-being. In this project model of visceral leishmaniasis caused by infection with *Leishmania donovani* or *L. infantum* and cutaneous leishmaniasis caused by infection with *L. major*, *L. mexicana* or *L. amazonensis* may be used. This allows delivery to cutaneous sites and the spleen, liver and bone marrow (visceral leishmaniasis) to be studied. There is no clinical vaccine to prevent leishmaniasis or many type of cancers, therefore another component of this project is to investigate the ability of novel vaccines given alone or with different immune stimulators (substances that enhance the immunity) to prevent infection. Different types of cancer may be used but the primary focus will be on using a murine model of lung cancer. This allows delivery to the lungs using different routes of administration to be studied with the primary focus being on non-invasive therapies. Cancer is a major health problem and there were an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020. Infections often modulate the host's immune response to enhance the survival of the pathogen or to allow the condition to progress in an individual. Therefore, the effect of treatment on host immune responses will be studied to give insights into what how host immunity is affected by cancer or leishmaniasis and to determine if it is possible to identify immunological markers that can be used predict successful treatment. In this project we will also study the ability of novel light treatment to treat cutaneous leishmaniasis as this may allow patients to self-treat their skin lesions and has the added benefit that it should make it less likely for parasites to develop resistance to this type of therapy as it does not use any drugs.

What outputs do you think you will see at the end of this project?

The primary output will be to publish the findings in high-ranking academic journals and at international conferences. This in turn will support applications for research funding. Work using novel drug candidates can help identify new compounds that have not been considered as anticancer or antileishmanial agents before and highlight these to other researchers. The identification of new drug candidates would be beneficial for both cancer and leishmaniasis. There are a limited number of clinical drugs for leishmaniasis and cancer and the use of the ones available are associated with adverse side effects, use multiple drug doses and their use is associated with the emergence of drug resistance. Achieving antiparasitic/anticancer levels at the site of infection or tumour cells is key to successful treatment and the ability to target the drug/drugs to this site is influenced by

how a drug is administered to a patient. Ideally non-invasive routes should be used as these would be more patient- friendly but this would usually mean packaging a drug in a delivery system. Understanding how the delivery system modifies the delivery of the drug is essential and we now have access to imaging systems that can allow us to track delivery to cancer/parasite cells and study their effect on parasite/tumour burdens in the same animal over time. There is no clinical vaccine recommended for leishmaniasis and identification of a suitable vaccine candidate would not only reduce the risk of people being infected but would also help global initiatives to eradicate the diseases. Leishmaniasis is also a veterinary problem so a vaccine candidate would also prevent infection in animals, particularly dogs. These animals can act as a reservoir of infection for visceral leishmaniasis. Studies completed in this project would also characterise the type of immune response associated with protection by determining antibody and cellular immune responses. This is important to know as it can help identify the type of immune responses that the treatment should induce in humans/animals and give specific markers to show if the treatments cause similar protective immunity in people/other animals. *In vivo* imaging studies from this study would be used to illustrate successful drug/vaccine efficacy and the role of internal processes in therapeutic outcome/susceptibility to disease. This can add to what is known about immune mechanisms and help other researchers understand immunological processes in these two conditions. In this project we will also investigate a novel light therapy for the treatment of cutaneous leishmaniasis. This type of treatment has the potential to remove the need for drug treatment for cutaneous leishmaniasis and could be instrumental in reducing the risk of drug resistance developing in these *Leishmania* species.

Who or what will benefit from these outputs, and how?

As this licence is for 5 years the long-term outputs are a contribution to the body of knowledge in the area.

Short term benefit: information for these studies would provide can be used to identify what effect changing a chemical's structure can have on anticancer or antileishmanial efficacy which can assist in rational design of compounds for other researchers. Novel carrier systems can provide a method to overcome some of the problems faced by other researchers using novel compounds e.g. solubility issues, ability to target to specific tissues in the body. The results from vaccine studies can assist in identifying the type of immune response associated with protective immunity and give important information on the effect of vaccine dose and dosing regimen on host immune responses. Phototherapy gives the opportunity to treat cutaneous lesions using a non-invasive method and can provide a method to combat drug resistant strains, as it will act via a different mechanism compared to current clinical drugs. There should be minimal risk of adverse effects because the wavelength of light we will use is less damaging than other wavelengths.

Midterm benefit: Data from these studies is of commercial interest and we seek patent protection for the intellectual property (IP) generated in studies. This can lead to collaborative studies or a licencing agreement with industrial partner to assist in the development of new anticancer drugs, or use of the delivery system for bespoke purposes, or development of a vaccine for leishmaniasis. The data from these studies will be published in scientific journals and presented at conferences so that the results can be shared with the scientific community. These studies will demonstrate the delivery systems used in studies which can act as platforms for other types of diseases. This would expand the impact of this research. In addition development of a vaccine for leishmaniasis would also develop technology that could be used for other conditions. For example, a production method has to be identified that allows larger scale manufacture of a recombinant protein

vaccine and this could be used for other recombinant protein vaccine. Leishmaniasis requires cell-mediated immunity rather than antibody based immune responses, therefore adjuvants that work in this model may also be suitable for other types of infections e.g. viruses, bacteria.

Long term benefit: The long-term goal of this project is to identify new and better treatments for leishmaniasis and cancer and thus ease the suffering of people who have these conditions, and reduce the mortality caused by these conditions. The results from these studies would not just be limited to cancer and leishmaniasis as the drug delivery systems could be used for other types of drugs.
Therefore, they can also be exploited to produce better formulations for drugs that treat other conditions
e.g. targeting a drug to the liver for liver cancer treatment or to the lungs for treatment of tuberculosis. The studies from this project would also benefit health care providers by reducing the number of patients requiring long term treatment. The ability to treat cutaneous leishmaniasis using light therapy would reduce drug use and stop any one from suffering from the adverse side effects caused by clinically used anti-leishmanial drugs, as all the drug currently used are associated with adverse side effects. A reduction in drug use would reduce the induction of drug resistance in endemic parasite populations and thus extend the clinical life of existing therapeutic drugs.

How will you look to maximise the outputs of this work?

I collaborate with colleagues within the University, in the UK and internationally to promote the research in identifying what immune responses are important in protecting against leishmaniasis, development of novel drug candidates for leishmaniasis and cancer treatment and investigating what factors are important in drug resistance in leishmaniasis. I also collaborate with companies in research projects to develop drug candidates against leishmaniasis and cancer and studying the effect of novel adjuvants on vaccine efficacy. The findings of our studies will be published in peer-reviewed journals for other researchers in the field and we will disseminate our findings through presentations at national and international conferences. Thus there are ample opportunities to share data and results with the international community. We also aim to promote public understanding of immunology, leishmaniasis and cancer via our outreach activities.

Species and numbers of animals expected to be used

- Mice: 1100
- Rats: 300
- Hamsters (Syrian) (Mesocricetus auratus): 550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Mice and hamsters are used as they provide well characterised animal models for *Leishmania* and cancer studies. Adult animals will be used as they have a fully competent immune system. Rats will be used for pharmacokinetic studies as they can provide a larger animal model and a larger blood/tissue sample for assessing drug levels after dosing compared to mice.

Typically, what will be done to an animal used in your project?

The exacts timings used in experiments may vary but an indication of the type of studies completed under this project licence is given below.

In drug studies *Leishmania* infected or animals given cancer and uninfected/normal controls will be treated with a drug formulation or vehicle alone by various routes e.g. intravenously, subcutaneously, orally, topical, intranasal, by inhalation. This may be a single treatment or multiple doses of a drug formulation. The animals will then be killed and the effect of treatment on parasite/cancer burdens determined. Most experiments are completed in 14 days but some experiments may continue up to 10 months so that the effect of treatment in chronic infections can be studied or to show that treated animals do not relapse. Animals may be imaged under general anaesthesia over the course of the experiment to determine burdens in the mice or assess internal processes e.g. recruitment of neutrophils or macrophages to the site of the infection, usually once/week. An imaging session usually last 15-20 mins for an individual animal and they recover within 10 minutes and show no ill signs from the procedure. Blood samples may be taken from animals at different time points.

In vaccine studies mice or hamsters are normally given two doses of a vaccine alone or with an immunostimulant by various routes e.g. subcutaneous injection, orally, intranasal, inhalation, normally with 2-3 weeks between immunisations. Animals are infected with *Leishmania* 2-3 weeks after the last immunisation and then humanely killed 2-4 weeks post-infection. The effect of vaccination on *Leishmania* parasite burdens is then determined. In phototherapy experiments mice with cutaneous leishmaniasis will be treated with light for a set period, normally less than 30 mins, and the effect of treatment compared to control determined. During experiments, animals may be imaged to monitor parasite/tumour burdens over the course of the experiment and to determine the effect of treatment on macrophages or neutrophils recruitment. This is usually once a week over the course of an experiment and an imaging session usually last 15-20 mins for an individual animal and they recover within 10 minutes and show no ill signs from the procedure. Blood samples may be collected at various times during the experiment from mice, usually once a week, to determine specific antibody titres after vaccination.

Hamsters and mice are also used to provide stock *Leishmania* parasites and these studies usually last 4-8 weeks for cutaneous Leishmania species (mice only) and usually 8-12 weeks for visceral leishmaniasis species (hamsters only). Animals are injected with the parasite once and then humanely killed when it is time to harvest the parasites for an experiment or if they show any signs of ill health before then.

In light treatment experiments, animals with cutaneous leishmaniasis are exposed to the light treatment under general anaesthesia so the animals keep still. The temperature of the skin is monitored to ensure that the animals do not get exposed to an amount of light that could damage the skin. The effect of light treatment on parasite burdens may be monitored by imaging the mice or by measuring the lesion size. Most studies would be completed in 3 weeks.

Rats used in pharmacokinetic studies are usually injected once with a drug formulation and then blood and samples collected from the same animal over a 24-hour period, or they may be killed at set times so that tissues can be harvested at different times. The animals usually show no signs of ill health.

What are the expected impacts and/or adverse effects for the animals during your project?

The effect of drug/vaccine treatment on animals will depend on how the drug is administered e.g. injection but it will cause transient pain that lasts a short time. Animals will be humanely killed before they harbour significant tumour burdens as imaging studies allow tumour burdens to be assessed from day 3 post inoculation for lung cancer studies. Most experiments using cancer cells are completed within 14 days. Blood samples will be collected from animals in some experiments to determine antibody levels but this is usually just once/week and will only cause transient, mild discomfort to an animal.

Phototherapy will be carried out under anaesthesia and may cause warming of the foot, but the temperature will be monitored to ensure it cannot cause a burn.

Anaesthesia should have no adverse effect on the well-being of the mice as the sessions are short and the frequency is kept within inhouse guidelines.

An imaging session to determine parasite/cancer burdens or internal processes e.g. recruitment of neutrophils or macrophages is usually last 10-20 mins for an individual animal and they recover within 5-10 minutes and show no sign of ill effects from the procedure. Injection of cancer cells to induce cancer is not associated with any obvious adverse effects on animal health and well-being as the imaging used can detect cancer cells before large or numerous tumours. Most studies only last up to 14 days for control animals. Experimental animals may be kept longer to show that a treatment cures an animal and it does npot relap

Animals with cutaneous leishmaniasis develop skin lesions (nodules), which are normally painless, even when sometimes they ulcerate. Animals will be killed if lesions have a diameter greater than 10 mm or have any sign of a secondary bacterial infection (usually manifested with pruritus and/or pus). Mice infected with visceral leishmaniasis do not normally shown any adverse signs due to infection, even at a year post-infection. Hamsters infected with visceral leishmaniasis will normally be killed at 8- 12 weeks post-infection, before they show adverse effects such as severe wieight loss of weigght gain (fluid retension). Nevertheless, all animals will be closely monitored and culled if they show any signs of ill health (e.g. weight loss, abdominal distension).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In most cases animals may be in the mild severity over the course of an experiment. However, it is not always possible to predict what effect a novel compound, formulation or drug combination has *in vivo*. In some cases *L. donovani* infection can be more severe effects in some hamsters and cause moderate effects, and this may reflect the outbred nature of the hamsters. There is no obvious effect in mice infected with *L. donovani* despite the fact that these animals have hepatosplenomegaly.

The adverse effect for each genetically modified mouse strain will be dependent on the specific genetic modification of the mice but it’s not expected to result in gross phenotypic abnormalities, the animals are expected to reproduce normally and mature to full age as wild type mice. The expected severity is mild (> 95%).

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?

Normal naive mice or genetically altered mice with modifications of specific genes or cells of research interest will be used in this study as they help identify what components of the immune response are important in controlling an infection. For example, we have shown that the cytokine interleukin 4 must be present for antimonial drug treatment to be fully effective. Certain strains of mice can be used as a host for *Leishmania* species and provide models of cutaneous and visceral leishmaniasis. For example, BALB/c mice are very susceptible to infection with *L. major, L. mexicana*, *L. amazonensis*, *L. donovani* and *L. infantum.* However Golden Syrian hamsters have to be used instead of mice to provide stock *L. donovani or L. infantum* parasites as mice cannot provide enough parasites. Many researchers use these rodent models as they have similar characteristics to human infections and they have been used in studies to develop clinical drugs e.g. amphotericin B formulations for the treatment of visceral leishmaniasis or in studies to identify vaccine candidates.

Mice will be used in cancer studies as they can be injected with luciferase-expressing cells that initiate a particular type of cancer. For example, mice given B16 F10 cell develop lung cancer and it is possible to monitor the cancer progression by imaging mice.

Rats will be used in pharmacokinetic studies as these animals allow a larger blood and tissue sample to be collected compared to mice.

Mice are the lowest vertebrate group where models of parasitic infection have been developed and refined and give repeatable results of low variability and high quality. The models used in this study have also been refined to reduce suffering to the minimum levels likely to give satisfactory result.

Only adult animals can only be used as the immune system of younger animals is not sufficiently mature to generate appropriate responses.

In all cases animals are required as the effect of treatment on multiple systems is required e.g. cellular and humoral immunity or we need to know the effect of a drug on a particular site and its effects on off target organs.

Which non-animal alternatives did you consider for use in this project?

We always screen any drug compounds *in vitro* before we perform *in vivo* studies so that only compounds with high efficacy and low cytotoxicity are used in *in vivo* studies. We screen the ability of serum from infected animals to recognise our vaccine candidates so that we know they can be recognised as part of a normal immune response and are

antigenic to the host. I have checked relevant databases such as AnimAlt-ZEBET or AltBIB and I have not found any useful alternatives for the intended animal studies.

Why were they not suitable?

At present there is no alternative that mimics the *in vivo* pharmacokinetics, the effect of other cells or cellular products on drug stability or site-specific activity as effectively as *in vivo* studies. The efficacy of a vaccine candidate depends on it being taken up by antigen presenting cells and the presentation of the antigen determinants to specific B and T cells within the body. Current immunological knowledge cannot predict exactly where this will take place and the influence other cell types, or what effect the infecting organism at challenge will have on the final immune response.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals will be used in each protocol below are based on current usage and potential research interests through collaborations.

Protocol 1: Identify parameters important in drug delivery. Hamsters 100, Mice 200, Rats 300 Protocol 2: Efficacy and immunological studies. Hamsters 300, Mice 800,
Protocol 3: Stock infections, Hamsters 150, Mice, 100

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This project licence will continue the studies from my current project licence PF669CAE8 and continues on from studies undertaken in project licences PPL60/3525, PPL60/3740, PPL 60/4252, and PPL 60/4334. Data from these studies gives data on drugs and their doses and will be used to design similar experiments. In addition to detecting significant differences between treatments at 80% statistical power and a 5% significance test level then a minimum of 6 animals/group are required if lung cancer is induced using B16 F10 cells, and previous experience has shown that we need a minimum or 4 animals/group in *Leishmania* studies. However in new studies using different study design online tools such as the NC3R's Experimental Design Assistant will also be used in designing studies.

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 using 3 mice/hamsters will be used when testing out a new entity e.g. drug compound/adjuvant where there is no available data on its *in vivo* safety. Animals used in this project are from commercial, approved or in-house breeding colonies as breeding animals are not part of this project licence. Sharing tissue from animals is routinely used e.g. to obtain bone marrow derived macrophages. I have developed novel luciferase-expressing *L. donovani* strains i.e. paromomycin resistant cell lines, miltefosine resistant

cell lines and antimony resistant cell lines. These are used to test novel drugs/drug formulations in *in vitro* drug screening studies. Unfortunately, these parasites have lost some of their infectivity and can only be used for short term experiments (approximately 14 days). Studies are still ongoing to try and increase their infectivity as they would be a very useful for *in vivo* studies and allow more data to be obtained in studies e.g. tracking parasite burdens over time in vaccinated mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In *Leishmania* studies both wild-type and luciferase-expressing parasites will be used. Luciferase- expressing parasites allow parasite burdens to be monitored in the same animal over time and this model results in lower parasite burdens in *L. donovani* compared to infection with wild-type parasites, so it is a less severe model as parasites can be detected much earlier post-infection. For example, in cutaneous *Leishmania* studies and cancer studies using luciferase-expressing cell lines it is possible to detect cells by day 3 post-inoculation so that it is possible to use a less severe model for screening drug compounds. Conventional methods would not allow lesions in the footpad to be detected until day 14 post-infection. This model also allows more data to be collected from animals as conventional methods only allow burdens to be determined by using callipers to monitor tumour size or lesion size (*Leishmania*) weekly.

Animals given cancer will be kept in barrier conditions so that any chance of acquiring airborne infections is minimised.

Animals will be housed in cages that have their environment enriched with houses and nesting material. Where possible animals are housed in groups so that animals are not housed alone but this is not possible if animals have to be separated due to fighting.

Why can’t you use animals that are less sentient?

Adult animals have to be used in studies because they have a fully mature immune system. *Leishmania* will only develop in certain animals and mice and hamsters are the two recognised models for this species. The cancer lines used in studies have been commercially developed for use in mice and the B16 F10 cell line homes to the lungs so that site-specific drug delivery can be studied.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most drug studies last 2 weeks and vaccine experiments are usually terminated 14-21 days after infection. Stock infections for cutaneous leishmaniasis are terminated before lesions reach a set size. Stock hamster experiments usually run for a maximum of 8 weeks

and they are terminated early if the hamsters show clinical signs. All experiments will be kept to the minimum length needed for the experiment to acquire the necessary data.

We use an improved administration protocol (e.g. preparing the injection site and needles, prior to administration of substances, carefully avoiding the leakage of injection reagents).

The use of *in vivo* imaging systems (IVIS) will allow us to determine disease onset at earlier time points and thus terminate procedures before the disease reaches a severe stage.

In cutaneous leishmaniasis studies we use footpad infections with luciferase-expressing parasites instead of rump injections as we can detect infections much earlier, probably because the parasites are in a small concentrated area. These parasites do not grow well in the rump so these parasites must be injected into the footpad to survive. I suspect the inserted gene makes these parasite less able to grow in the skin.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance from the NC3Rs of animals in research, LASA good practise guidelines, and advice from RSPCA for laboratory animals, and in-house guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I currently receive the NCR's newsletter about new developments and I will learn advances in the 3Rs through various opportunities such as the University annual 3Rs symposium.

105. Cancer development, growth, detection and treatment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Angiogenesis, Metastasis, Translation

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Most solid cancer related deaths are a result of resistance to therapy and metastasis. Identifying patients who have a high risk of progression and determining alternative strategies to treat them is required. Exploring the molecular mechanisms that promote resistance and metastasis at different stages of the disease may provide the best potential targets for therapeutic intervention.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This research will inform us better about human cancer and how to detect and treat it efficiently, and, in the future, allow us to test potential new treatments. In recent years it has been established that cancers that develop in different organs, behave differently thus requiring individual treatment strategies. By understanding the cellular and molecular mechanisms that drive tumour growth and metastasis in different cancer types we can identify new strategies to treat cancer and prevent millions of deaths each year.

What outputs do you think you will see at the end of this project?

Our proposed studies are directly relevant to improving our understanding of the mechanisms of cancer progression, resistance to therapy and metastasis, possibly indicating improved identification of patients with increased risk of progression. These studies should also result in the publication of peer reviewed papers in high impact journals.

Who or what will benefit from these outputs, and how?

By making target discoveries in human cancers and testing these in mouse models of cancer, our research will have clinical relevance.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, we have continued support of postdoctoral fellows and the technical expertise required for the research. Importantly, this research has been built around a team of internationally recognised co-investigators and collaborators who have helped design the research and will provide clinical, scientific and technical expertise to support its success.

Species and numbers of animals expected to be used

- Mice: 32,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Tumour growth and spread is a complex process involving several cell types and different cellular interactions. This complexity cannot be recapitulated in any *in vitro* system or lower organismal system such as flies or fish, hence the need to use mice for this research. Although it is possible to use lower organismal system such as flies or fish to study cancer, the mouse has many similarities to humans regarding anatomy, physiology and genetics which is lacking in flies and fish. Around 90% of the genes in mice are the same as in humans, making them the ideal model for studying cancer and its spread. Mice are also better for studying complex biological systems found in humans such as the immune system, playing an important role in fighting cancer, which is more primitive in fish and flies. The majority of experimental mice will be used between 3-6 months old. This is because prior to 3 months, mice are continually growing rapidly, but this stops at around 3 months. After 6 months of age the maturation rate of mice is 25 times faster than humans. Therefore between 3 and 6 months, the mouse is considered to be mature and not yet

aged. After 6 months, cell senescence and other age-related issues may affect our biological readouts.

Typically, what will be done to an animal used in your project?

A typical experiment follows the described steps below :

Injection of tumour cells and/or injection of chemical to induce deletion of candidate genes. This may include surgical procedures for injection of tumour cells directly into the organ of interest.

Monitor tumour growth using manual measurements (Caliper) or minimally invasive imaging such as Magnetic Resonance Imaging (MRI).

Administration of chemotherapy drug (injection) or radiotherapy.

Monitor tumour growth using calipers or minimally invasive imaging until tumours reach reach humane endpoint established in this license or mouse shows signs of clinical sickness.

Examine primary tumour growth and metastasis to distant organs.

Depending on the mouse model, experiments can last for between 2 weeks - 4 months

The number of procedures varies from 1 to 30 (e.g. for minimally invasive dosing of therapeutic treatments), depending on the experimental design.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects are unavoidable but are not expected to be severe. Adverse effects due to surgical and genetic interventions that are necessary to achieve the objectives, and adverse effects associated with tumour growth in our untreated control animals, will be minimised using a careful and considered monitoring approach, particularly where the animal begins to show any signs of disease.

Possible adverse effects may include weight loss, digestive impairments, pain after surgery, or abdominal pain linked to pancreatic tumour growth or early ulcerations of subcutaneous tumours. The duration of these effects will be minimised by close monitoring of the animals and setting up clear humane endpoint to avoid any progression beyond temporary moderate severity. . Any animal showing any of the above symptoms/signs, although likely to occur very rarely in well-designed experimental studies will be killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the severities will be mild to moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Tumour growth and spread is a complex process involving several cell types and different cellular interactions. This complexity cannot be recapitulated in any in vitro system. Although it is possible to use lower organismal system such as flies or fish to study cancer, the mouse has many similarities to humans regarding anatomy, physiology and genetics which is lacking in flies and fish. Around 90% of the genes in mice are the same as in humans, making them the ideal model for studying cancer and its spread. Mice are also better for studying complex biological systems found in humans such as the immune system, which plays an important role in fighting cancer, which is more primitive in fish and flies.

Our research group has a very strong history using animals in cancer research, so our choice of mice is the simplest mammal that through years of research by ourselves and others provides us with confidence that our experimental designs are likely to be as close to our therapeutic goals as possible. This will allow us to achieve a successful project, described in ths application.

Which non-animal alternatives did you consider for use in this project?

Although we cannot use non-animal alternatives to study tumour growth and metastasis, at all stages throughout the project simplified models for cell growth in culture, culture of an organ collected from an animal assays or assays where tissue is removed from the animal to examine blood vessel growth in culture will be used. We will also utilise established cell lines and primary cells. These will help to identify the molecular mechanisms that underlie the in vivo characteristics of cancer growth and spread. Additionally, we have access to human tissue banks to obtain human tissue to examine cellular responses during tumour growth. This will help to clarify the relevance or our results to human physiology and pathology.

Furthermore, to avoid duplication of in vivo work we will consult both the IMPC (International Mouse Phenotyping Consortium) and KOMP2(Knockout Mouse Program) prior to undertaking any in vivo studies.

Why were they not suitable?

Non-animal alternatives are not wholly suitable because tumour growth and spread is a complex physiological process involving several cell types, complex interactions between different cell types and de-regulation of hundreds of molecules. Furthermore, tumour growth is a dynamic process which changes as the tumours grow larger.Therefore, this complexity cannot be recapitulated in any in vitro system. Although it is possible to use lower organismal system such as flies or fish to study cancer, the mouse has many similarities to humans regarding anatomy, physiology and genetics which is lacking in flies and fish. Around 90% of the genes in mice are the same as in humans, making them the ideal model for studying cancer and its spread. Mice are also better for studying complex

biological systems found in humans such as the immune system, which plays an important role in fighting cancer, which is more primitive in fish and flies. Thus the utilisation of mice for in vivo experiments is essential to our proposal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sample size calculations will be performed before each experiment so that experiments are adequately powered statistically, in consultation with our in-house statistician, Dr Adam Brentall. Many of these are already done for the experiments planned in this proposal. Using the appropriate numbers of animals is essential to answer reliably the questions we are addressing. This ultimately avoids wasting animals and repeating experiments unnecessarily. To further limit animal wastage we will utilise the optimum breeding strategy to minimise over-production of animals as well as only breeding the numbers of mice required to produce the correct numbers of animals needed for our experiments. We have a dedicated named colony manager who oversees our colony management.

We will share our animals/tissue resources with other research groups within the Institute to maximise outputs. We will also maximise the amount of information we obtain from each animal by harvesting all tissues.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experimental protocols will include the following:

clear aim

detailed description of the experiment including treatment regimes, experimental groups and experimental material to be collected and endpoints

outline of the method of analysis of the results

blinding and randomisation of experimental groups will be used to avoid bias

Imaging technologies will be a vital tool in reducing the number of animals required for each experiment as this removes the need to kill animals at time points to observe tumour progression.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All experiments will be carried out following good laboratory practice. All experiments will be designed after careful examination of the literature, with regards to treatment ie. drug dosage, treatment regime. Pilot studies will also be performed to help refine our

experiments and determine the variables that can impact upon experimental results and the sample sizes required to accurately determine statistical significance. We will also use PREPARE guidelines when planning experiments (https://norecopa.no/media/7832/prepare-guidelines.pdf).

When reporting on *in vivo* experiments for publication we will conform to the ARRIVE guidelines to minimise unnecessary studies.

We will archive genetically modified mouse lines as frozen sperm or embryos to reduce the number of living mice we have to maintain and to allow sharing of these lines between researchers, providing further opportunity for reduction.

Imaging technologies will be a vital tool in reducing the number of animals required for each experiment as this removes the need to kill animals at time points to observe tumour progression.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically Modified Mice (GEMM) will be used. Some of these mice will naturally develop cancers of interest (including pancreas, breast and lung) so that we can investigate the biology of the developing disease and test our treatments in a model that most closely matches human diseases. For most models the mice do not develop any pain or discomfort as a consequence of their developing cancers and we terminate the animals before such discomfort occurs due to tumour size. MRI monitoring of deep tissue tumours provides confidence we can deliver this intention.

Mice injected with transplanted tumour cells/tissues will also be used: Cells or tissues derived from mouse or human tumours will be injected or implanted into mice via various routes including subcutaneously (sc), intravenously (iv) (to examine metastasis), intraductal into the mammary fat pad, as required by the scientific question. Injections will be carried out by experienced handlers thus minimising pain and discomfort to mice. Mice will require anesthesia and surgery for orthotopic injections and implantation of organoids and will receive analgesia post-op to minimise discomfort. In some cases genetically manipulated cells that contain bioluminescence or fluorescence will be used so we can accurately monitor tumour development and response to therapy using non-invasive methods so as to minimise pain and harm to animals.

All experimental mice are routinely examined for their health status and any behaviours or appearances that suggest pain or discomfort will be monitored closely with the personal licencees, the local BSU staff and as required, the NACWO and NVS.

Why can’t you use animals that are less sentient?

Most of our experiments require that tumours will develop over a time period. Tumour growth requires the complex interaction between different cell types that cannot be recapitulated in less sentient animals. Because of this, it is also not possible to use animals that are kept for a long term under terminal anaesthesia. We also need to closely reflect as much as possible the biological processes in humans that we are trying to investigate, which can be best mimicked in mice. Use of non-mammalian models (drosophila, xenopus, zebrafish) would not recapitulate the biology that occurs in humans. Our choice of mice is the simplest mammal that through years of research by ourselves and others provides us with confidence that our experimental designs are likely to be as close to our therapeutic goals as possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Imaging technologies, such as MRI, are a vital tool in refining the number of animals required for each experiment as this removes the need to kill animals at time points to observe tumour progression. It also allows us to recruit mice that have a similar tumour volume at the start of an experiment.

We already are successfully implementing MRI and other imaging methods (high resolution ultrasound) at our Cancer Institute to improve the analysis and monitoring of deep tissue cancers that occur either through orthotopic tumour growth in transgenic mice. Such imaging techniques have the immediate benefit that there is limited chance that an animal develops an internal tumour that exceeds home office limits. This has also improved how we apply experimental therapeutics as we can now 'recruit' mice that have similar volume tumours at Day 1 of a protocol, instead of starting therapy on a cohort at a predetermined time interval after injection. This also allows longitudinal studies that formally required cohorts of mice to be killed at intervals, thus saving many mice.

By internal talks and eventual publication we are sharing these improvements with our local and wider scientific community.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines outlined in the manuscript "Guidelines for the welfare and use of animals in cancer research" Workman et al; 2010. We also follow the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines), PREPARE guidelines (https://norecopa.no/media/7832/prepare-guidelines.pdf) and UKRI guidelines (https://www.ukri.org/about-us/policies-standards-and-data/good-research-resource-hub/use-of- animals-in-research/).

We will take advantage of the free Experimental Design Tool (https://eda.nc3rs.org.uk) when designing animal experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will have regular discussions with the NVS, NACWO, NIO, the AWERB and animal technicians regarding our current practices and whether we need to review these. We will follow all the guidelines from the following;

https://www.ukri.org/about-us/policies-standards-and-data/good-research-resource-hub/use-of- animals-in-research/

https://www.cancerresearchuk.org/about-us/we-develop-policy/our-policy-on-supporting-science/the- use-of-animals-in-cancer-research

We will also make use of NC3R webinars, events and workshops as and when necessary.

106. Breeding, maintenance and supply of Genetically altered and harmful mutant 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
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

No answer provided

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo
Rats	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project is to breed and maintain high health status rats and mice with genetic alterations and harmful mutations and to supply them for research into the control of disease, ill health or abnormality and/or the study of normal and abnormal physiology, biology or behaviour. They will be used for the discovery and development of new medicines for the treatment and prevention of 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 use of GAA has grown worldwide over the years, not only have new lines been created but also as new uses are being discovered for existing lines.

Similarly, many of these lines are of interest to many different research groups and to have a single source with the ability to distribute to worldwide locations reducing the number of breeding colonies required, in turn reducing the numbers of animals produced and minimising waste.

Many establishments do not have the facilities, equipment or expertise to import, breed or maintain GA colonies which results in the continued international transportation of large experimental groups. Where appropriate the provision of importation, animal facility space and colony management/breeding services may improve animal welfare by removing the need to regularly transport large numbers of animals over considerable distances.

The maintenance of high health status animals also ensures that high quality animals are produced, improving the quality of experimental data and scientific results and reducing the number of animals required. In having the ability to re-derive lines we aim to improve animal welfare standards and breeding efficiency by removing contaminants that are detrimental to research, animal facilities and breeding performance and maintain an availability of high health status animals removing the necessity to breed lower health status animals elsewhere. As the project is for the provision of services and supply of genetically altered and harmful mutant rodents, we do not benefit from the scientific work undertaken on the animals bred or distributed.

What outputs do you think you will see at the end of this project?

The supply of our genetically altered rodents has already contributed to the development in vaccines for dengue fever more recently there has been a need to study the aetiology of the Zika virus, and our strains of mice have fitted this market well. It is clear that IFN-α/β plays a significant role in preventing viral replication and protecting against Zika virus disease.

The continuation is supply would further assist in scientific insight underpinned by this project and will lead to a greater understanding of these viruses ,research publications and advances in the prevention of viral diseases of man.

Who or what will benefit from these outputs, and how?

The Supply of transgenic mice will continue help enhance knowledge in developing vaccines for areas of research such as dengue fever and Zika virus, our transgenic mice are also continual uses within virology, oncology, cardiovascular and pulmonary diseases, inflammatory and immunological diseases and neuropsychiatric disorders will aid in the discovery and development of new medicines for the treatment and prevention of human disease.

How will you look to maximise the outputs of this work?

The purpose of maintaining a breeding colony is to supply a range of customers around UK and the rest of the world, who are working on various research involving our specific GAA rodent strains. We will collaborate on a supplier level and gain insight into future demand, along with continued forecasting updates to allow for the continued supply.

Species and numbers of animals expected to be used

- Mice: 125000
- Rats: 50000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Genetically altered rodents are currently in widespread use in biological, medical and veterinary science and have shown to be of great value in interpreting the function of genes and pathways in a wide variety of biological, physiological and pathological processes.

To supply genetically altered rodents, the chosen life stages for the breeding and maintenance by means of conventional breeding methods are required to enable us to continue and add value in these areas.

Typically, what will be done to an animal used in your project?

Animals bred and maintained on this project will be used for continued use in breeding programmes by conventional methods and supply of stock animals, for scientific use or distributed for use on other project licences within the UK or in bona fide research establishments outside of the UK.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals produced for this project license are not expected to exhibit any harmful phenotype, and thus are not expected to exhibit pain, weight loss, tumours, or abnormal behaviour.

Any animals exhibiting any unexpected harmful phenotypes will be killed in by an appropriate method accordance to Schedule 1 of the A(SP)A.

Expected severity categories and the 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 likely/expected level of severity for breeding and maintaining the colony will be mild.

Retired breeding stock and any animals exhibiting any unexpected harmful phenotypes will

be killed in by an appropriate method accordance to Schedule 1 of the A(SP)A.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Use of live animals is required for breeding purposes and as embryo donors and recipients.

Which non-animal alternatives did you consider for use in this project?

Cryopreservation and the archiving of embryos will remove the need to maintain live colonies.

Why were they not suitable?

Live Animals are required for continued use in breeding programmes and the supply of live mice to establishments within the UK and worldwide who do not have the facilities, equipment or expertise to import,breed or maintain GA colonies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experience of supplying Genetically altered rodents under previous Breeding, maintenance and supply of Genetically altered and harmful mutant rodents project licences, has given insight into future demand, this along with continued forecasting updates will allow breeding programmes be maintained to optimise breeding levels in order to reduce numbers of animals produced and minimise wastage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Long standing experience and the use of proven protocols for the supply Genetically altered rodents under previous project licenses, has given insight into optimal numbers required to maintain colonies and ensure minimal requirements.

What measures, apart from good experimental design, will you use to optimise the

number of animals you plan to use in your project?

Breeding programmes will aim to optimise breeding performance in order to reduce numbers of animals produced and minimise wastage.

Production data relating to animal breeding performance will be collected and analysed on a regular basis to determine production targets, assess customer demand and to monitor and reduce any wastage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice will be used for the breeding, maintenance and supply to bona fide customer establishments. Standard protocols, methods and reagents have been optimised for this species and there are acknowledged benefits from their use. Rats are less commonly used but their larger size may make them a more suitable model if surgical interventions are required or historical/background data is predominantly in this species.

All animals will be regularly observed for signs of ill health or distress, with the appropriate action being taken as required. Records will be maintained of all strains held detailing their husbandry requirements and data will be collected on the clinical, behavioural and welfare effects of the transgene in these animals. This information will be made available as appropriate. Animals with clinical, behavioural and welfare effects will not be kept without prior consultation with the Home Office.

Why can’t you use animals that are less sentient?

Live Animals are required for continued use in breeding programmes and the supply of live mice to establishments within the UK and worldwide who do not have the facilities, equipment or expertise to import,breed or maintain GA colonies

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The specific details of each line maintained will be determined by current demand and trends but also by individual customer requirements.

Only animals of a high health status will be bred and to ensure that high welfare standards are maintained background information relating to the origin, health status, genetic status, welfare and husbandry requirements of each line will be obtained. Animals will be housed in accommodation designed to maintain their health status and prevent infection.

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

Animal breeding will operate under guidance and in accordance with The Animals (Scientific Procedures) Act 1986 (ASPA). As well as a guidelines and recommendations provided by NC3R's website such as Colony management best practice and Breeding and colony management.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular review of guidelines and recommendations provided by the recent reports on the NC3R's website such as Colony management best practice and Breeding and colony management will be sort. Any advances will be discussed with the Animal Welfare and Ethical Review Body (AWERB) for implementation and reviewed at recorded AWERB committee meetings to determine effectiveness.

107. Genes and environment at the interface of inflammation, immunity and metabolism

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Inflammatory bowel disease, Immunity, Metabolism, Cancer

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to elucidate how risk genes of human inflammatory diseases operate, and how they contribute to disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Enormous efforts have been made to identify inherited risk factors of disease. The inflammatory bowel diseases Crohn's disease and ulcerative colitis have been amongst the most successfully studied in this regard, with now >250 spots in the genome identified that increase risk for disease. What is often overlooked is that only a handful of those identified genomic loci are biologically well understood, meaning that it is known how they mechanistically contribute to disease. For some of these risk loci, not even their basic function is known. Our group has a long track record in deciphering major mechanisms of Crohn's disease and ulcerative colitis by studying genetic risk factors. As examples, by studying specific genes, we have defined how two cell biological processes, called

'endoplasmic reticulum stress' and 'autophagy' contribute to the disease process. In this study we will investigate how further IBD-associated genes, which we have identified as intricately linked to cell metabolism, contribute to disease pathogenesis.

Variants of a number of risk genes predispose for Crohn's disease and ulcerative colitis, two inflammatory bowel diseases; for primary sclerosing cholangitis, a chronic inflammatory liver disease; Still's disease, a childhood fever syndrome with debilitating arthritis; and leprosy, a chronic bacterial infection of the skin. They hence predispose for major human diseases spanning 'autoinflammation' and 'autoimmunity', meaning diseases where the immune system appears to attack the body and organ. Ulcerative colitis and primary sclerosing cholangitis are also the diseases with the highest risk of developing inflammation-associated cancer.

Our studies will identify key mechanisms of disease, a prerequisite to develop effective treatments. All of these conditions remain poorly understood, and for some of them, such as primary sclerosing cholangitis, there is not even a single approved medical treatment available. Insight gained from our studies are very likely to have ramifications way beyond the diseases the risk genes are associated with, given how fundamental for cellular energy metabolism their function is.

Studies such as ours are key to fill 'risk coordinates in the genome' with 'functional life' - a prerequisite to allow for the genomic revolution in medicine, an area where the UK is world-leading, and one that is the very focus of the government's life sciences strategy.

What outputs do you think you will see at the end of this project?

We will generate new insight into the major biological processes underlying inflammatory diseases. We will publish these insights in scientific journals, and present them at conferences.

Who or what will benefit from these outputs, and how?

Projects such as ours are the very basis for future therapeutics. Patients suffering from Crohn's disease, ulcerative colitis, primary sclerosing cholangitis, Still's disease and leprosy, as well those suffering from cancer, will ultimately benefit from these outputs. Mechanistic insight into major disease processes has been the basis of breakthrough treatments for these diseases - examples are new dugs that target inflammatory mediators called Interleukin-12 and Interleukin-23. We expect very similar outcomes and hence benefits from our research here, too.

More immediate beneficiaries will be the scientific community, both in academia and in industry. This research will advance insight into major disease processes. Given the specific focus, there is also a high likelihood that insight gained through this programme will be important well beyond the inflammatory diseases the genetic association is with. Specifically, we think that outputs from this programme will be important for researchers working to understand cancer and metabolic diseases.

How will you look to maximise the outputs of this work?

We extensively collaborate and share new results early-on. Large data-sets will be deposited at databases for other scientists to use and exploit. We present our work at conferences prior to publication. We often report on unsuccessful approaches and non-significant data, too, since these are often as valuable as successful experiments and their

results. We typically publish our work in major journals, which maximises the visibility of our work.

As we have in the past, we will seek patent protection where and when appropriate. This enables the development of new therapeutics, exploiting our discoveries.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most appropriate animal to investigate human genetic risk factors of disease. In the mouse, genes can be altered, which allows us to alter those that are involved in human disease. The immune system and metabolism are very similar between mice and man, and our understanding of mouse physiology is very deep. There are also many experimental tools, such as genetic models, recombinant cytokines (these are molecules that signal between cells) and antibodies, available for mice.

We investigate risk genes for human autoimmune/autoinflammatory diseases. The risk genes we are interested predispose for the inflammatory bowel diseases Crohn's disease and ulcerative colitis; a childhood arthritis with recurring fever that is called Still's disease; the liver disease primary sclerosing cholangitis; and the chronic bacterial infection leprosy.

All of these diseases arise from a complex environment - gene interactions. They involve numerous different cell types and organs. An example is the gastrointestinal tract, which contains many different cell types in a particular architecture within the organs. These include the intestinal epithelium, which is the inner barrier of the body, and which itself is composed of several different cell types; multiple types of immune cells, such as macrophages, dendritic cells, T and B lymphocytes; fibroblasts, endothelial cells (cells that cover the inner lining of vessels), neurons, etc. They are all involved in the disease process. The intestinal tract also contains the intestinal microbiota, an enormous accumulation of diverse bacteria, viruses and fungi that actually outnumber the cells of the human (or mouse) body. Over the last decade it has emerged that the microbiota has a huge impact on many diseases, including a particular large one on those we are studying.

Unfortunately there are no cell culture systems in a dish that come any close to modelling this vast complexity both on the host side, and on the microbial side.

Regarding life stage, we will generally study adolescent and adult mice.

Typically, what will be done to an animal used in your project?

We will generate, breed and maintain genetically-altered mice in this project.

Almost all of the mice we generate will carry genetic alterations. These genetic alterations are typically not harmful or even noticeable, and mice breed and develop normally.

Only few mice will carry mutations that lead to spontaneous phenotypes, specifically mutations that result in tumour development.

Small tissue samples will be taken to determine the genotype of mice. This is typically done at an early age (3-4 weeks).

Most genetically altered mice will not have *any* further interventions performed on them. They will serve as donors of tissues or cells, commonly harvested at an age of 8-16 weeks. Cells and tissues will be obtained from dead animals or during terminal anaesthesia. Most of our investigations will be reliant on primary cells and tissues obtained from these mice. Cells and tissue will then be studied in the laboratory in primary cell cultures in a dish, or analysed for gene or protein expression, or for metabolites.

Only a minority of animals will undergo further procedures:

In some mice, their metabolism will be characterised. This may require housing them for 2-5 days in special cages that monitor energy consumption. Occasionally, single mice will be housed in individual cages. Few mice will be fasted for up to 18 hours, and small amounts of blood will be taken from the tail vein at several time-points after metabolic 'fuel' (e.g. glucose) has been administered.

Some mice will be housed in special isolators that prevent colonisation with microbes. Such 'germ-free' mice generally develop normally. They may then be colonised with specific microbes, which are typically administrated through the mouth via oral gavage.

Mice may also be injected with agents, administered into the tummy (i.e. into the 'peritoneum') or underneath the skin. Or agents may be administered through the mouth via drinking water, food, or per gavage; or via the nasal route. Agents may include substances such as drugs, metabolites, immune- stimulating reagents. They may also include microbes such as viruses or bacteria. Colonisation experiments may last several weeks, infection experiments typically 1 to 4 weeks, depending on the agent.

On few mice we will conduct imaging studies, such as computer tomography. This may require administration of imaging agents.

Depending on the route of administration and type of agent, we may apply a local or general anaesthetic prior to the agent.

Some animals will be injected with tumour cells to study tumour growth and how this can be treated. These experiments typically last 3 to 4 weeks.

Very rarely we will induce overt disease in mice by administering agents that cause colitis (inflammation of the gastrointestinal tract) or arthritis (inflammation of the joints). The duration of these models varies from few days to few weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice will not experience more than mild and transient discomfort. This is due to when a tiny piece of tissue is taken for genotyping.

Most of the genetic alterations we will study do not cause spontaneous harmful phenotypes.

Mice prone to develop intestinal tumours may stop gaining weight or may lose weight. Tumours typically develop slowly over many (12-20) weeks.

Administration of immune-stimulating reagents, infectious pathogens or colitis/arthritis-inducing agents can cause abnormal behaviour, reduced food intake, pain and weight loss. The duration of clinical symptoms varies between models, typically lasting from few days to few weeks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of mice will fall under the 'sub-threshold' or 'mild' severity banding.

A minority of animals will develop 'moderate' symptoms. We will not pursue experiments that would fall under the 'severe' banding.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our research programme uses a wide range of approaches to elucidate disease mechanisms. These range from the atomic/molecular (e.g. structural biology), protein-based (e.g. enzymology), to cell culture in a dish. The latter includes 'permanent' cell lines (typically meaning cancer cell lines that can indefinitely be grown in a dish), genome-edited human pluripotent stem cells (iPSCs), and the *ex vivo* experimentation with 'primary' cells and tissues that are isolated from mice.

For every question we ask, we always use the simplest model system that is available.

We only use animals (mice) when there are no other suitable models available to answer specific questions that arise from our overall research programme. In fact, research involving animals are only a small part of our overall research programme into mechanisms of human disease. We use them judiciously and carefully when no alternatives exist to answer an important question.

The very nature of the biochemical mechanisms we investigate limits the use of *permanent* in vitro cell cultures. This is why we have to rely in these instances on 'primary' cells (meaning, these are taken out of a living organism - e.g. from humans or mice). It is important to appreciate that the proteins we study are 'polymorphic' in the human population, meaning the protein sequence differs at specific amino acids between

individuals. To demonstrate what the consequences of this variation is, we have genome-edited the corresponding parts of the mouse genome. This allows us to study them on an identical genetic background.

Many aspects of human disease processes cannot be modelled *in vitro*. This is very apparent for intestinal inflammation, which involves multiple different cell types (various T and B lymphocytes, macrophage and dendritic cell types, innate lymphoid cells, natural killer cells - these are all immune cells; fibroblasts, epithelial cells and their subtypes, endothelial cells, neuronal cells, and many others) and involves complex interactions with the intestinal microbiota, the enormous amount of diverse bacteria (and other forms of life) in our intestine that actually outnumbers the actual human cells. Neither the host side, nor the microbial side can be modelled *in vitro* to any meaningful extent. Whilst a microbiota involvement might be most obvious for intestinal inflammation, it has become very clear that it plays a similarly huge role for inflammation at other sites, for the organisms' metabolism, and may even determine the effectiveness of differnt types of cancer treatment.

The extremely high conservation between mouse and man of the genes and pathways we study, and the close similarity of the mouse and human immune system and metabolism in general render the mouse the most appropriate model system. Finally, the biochemical mechanisms we have discovered and that underpin our research programme have huge ramifications for organ and inter-organ physiology. Again, this could not be studied in a more reductionistic system than an in vivo model.

Which non-animal alternatives did you consider for use in this project?

Mouse experimentation forms only a small part of our overall research programme into human disease mechanisms. Our mouse work is embedded in a much larger research programme that uses a wide variety of technology.

We are using structural biological methods, protein chemistry, enzymology, metabolomics, and various other approaches to gain fundamental insight into mechanisms of disease. These are methods that let us study the proteins themselves that we are interested in. They let us often study even single atoms and help us understand how these proteins work. These studies form a central and large part of our overarching research programme.

At a further level of complexity, we want to understand how these proteins operate within a cell. For this we use cell culture in a dish. We employ permanent cell culture of various (cancer) cell lines (these have names such as MODE-K, Caco2, HEK293, THP1, B16, etc), as well as complex 'organoid' cell cultures (these are 3D cell cultures that can resemble the basic architecture of the cell type that is characteristic of a specific organ), including those derived from human induced pluripotent stem cells (iPSCs). We have experience in using gene-editing techniques to edit the genome of human iPSC to toggle the put the human gene variants into their genomes. Such 'toggling' is important to study what consequences a specific human variation has.

We also perform studies with cells isolated from peripheral blood from healthy individuals. The latter allows us to identify individuals who are homozygous for either variant of the polymorphic genes we study.

Experimentation involving primary cells from genetically-altered mice, and *in vivo* experimentation in mice form only a small part of our overall research programme and are used when absolutely necessary.

A large part of our work involves the activity of the immune system. Since the molecules that present antigens are highly polymorphic (this means they are different between every human) makes many studies of antigen-specific immunity impossible in cells from cohorts of humans - even when disregarding that the site of primary immune-stimulation is in tissue which would generally not be accessible for experimentation in humans.

We have extensive experience in all of these methods and use them on a regular basis. We constantly re-assess what the most appropriate experimental approach is.

Why were they not suitable?

As already alluded to above, many aspects of human disease processes cannot be modelled *in vitro*. This relates to the variety of cell types involved; the environmental signals/cues; the microbiota and many others. Many relevant cell types are not accessible at all in e.g. peripheral blood from humans. An example of the challenges we are facing is the intestine. While 'organoids' are useful model systems, these are clearly not mini organs. For example, intestinal organoids show a 3D structure reminiscent of the intestinal epithelium (the cell type that forms the single-layer cellular interface with what is inside the lumen of the intestine, meaning the enormous amount of bacteria alongside the food that is being ingested and digested), but they do not contain the myriad of immune cells, muscle cells, vessels, fibroblasts and neurons that form our intestine. They clearly also do not contain the unique ecosystem of bacteria, viruses and fungi that is present inside our intestine.

A particular challenge in our case is that the proteins we are studying are polymorphic, hence we would need to 'pick' healthy individuals for homozygosity of the risk and non-risk genotypes. We have indeed conducted select experiments with the support of individuals, but for reasons that are obvious these individuals could not possibly supply our research programme with a continuous supply of primary cells. Since a large part of the project involves studies of the immune system and the antigen presenting molecules and the receptor that recognise these antigens differ from person to person (they are highly 'polymorphic'), it would be impossible to find individuals that have either of the variants of interest, but are identical for the immune receptor.

Finally, the very nature of the biochemical mechanisms we investigate limits the use of *permanent* in vitro cell cultures, albeit we use them extensively whenever this is possible. These in vitro models also have obvious major advantages for mechanistic studies, due to the simplicity of their pharmacological or genetic perturbation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

By keeping our experimental conditions well controlled we are able to perform highly reproducible and statistically meaningful experiments using the minimal number of

animals. The experimental approaches described herein have been vigorously evaluated over the past two decades, thereby providing us with a wealth of data that help us optimise experimental design and achieve robust data from the minimal number of mice used. We have extensive experience in experimental design and statistics. Post-doctoral scientists in our group have been on experimental design courses to help understand sources of bias and variation and how best to reduce them. We also have access to external advice on statistics, if required, to help guide experimental design. Where possible in our design we blind people to genotype/treatment groups with different people doing infections or analysis.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For our various experimental approaches, be it *in vitro* experimentation on primary cells, or *in vivo* experimentation in particular models, we have extensive data from various contexts. These insights are invaluable for designing robust experiments with the minimal number of mice required. We have used the NC3R's experimental design assistant for work we have done on previous studies, and will continue to use it in future studies. The PREPARE and ARRIVE guidelines have been consulted for formulation of this project, and these will be followed to ensure continued communication between animal facility and our team, and reporting data from our experiments. We also collaborate extensively, seeking input and advice from colleagues who are experts in particular models.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When required and as part of this programme, we will use pilot studies to inform us on the control experimental conditions we need to use. We have extensive experience in the *in vivo* inflammation, infection and tumour models in this project, and thereby access to a wealth of data that make predictions of effect sizes and optimisation of experimental strategies easier. In an iterative way, we are thereby able to continually improve and optimise our strategies.

Please also note that these experiments involving genetically-altered mice is embedded in a much larger research programme that uses a wide range of different techniques to elucidate mechanisms of disease. These range from 'atomic resolution', using structural biology approaches, to enzymology, assays on recombinant proteins expressed in various in vitro expression systems, to a wide range of cell culture work, also involving human induced pluripotent stem cells that are being genome-edited. It is a typical feature of our programme that results from e.g. structural or enzymological experiments raise hypotheses that trigger experiments in cell culture or in cells from genetically-altered mice; and *vice versa*. This integrated, comprehensive and iterative approach allows for very judicious use of animal experimentation, with the constant aim to reduce the numbers of mice needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The vast majority of genetically altered mice generated on this license will not undergo any procedures apart from genotyping, hence will not experience pain, suffering, distress or harm. These mice, once dead or terminally anaesthesised, will serve as donors of primary cells and tissues. The genetic modifications we study do typically not cause spontaneous phenotypes.

A small minority of mice will undergo procedures. These include adminstration of reagents or cells; infections with pathogens; induction of inflammation, such as colitis and arthritis; and induction of tumours. We believe that the similarities in the mouse and human genomes are such that we can infer between the two, and we have closer links than ever before with patients suffering from these conditions. Over the years we have gained tremendous experience with our models and, through careful observation, we are able to minimise the potential suffering of the animals. We have been able to identify key clinical signs that indicate illness and consequently such animals can be quickly and humanely killed.

Why can’t you use animals that are less sentient?

As noted above, most of the mice generated on this license will be used as organ, tissue or cell donors when they are dead, or after terminal anaesthesia.

It is not possible to model all aspects of tissue inflammation, such as in arthritis or colitis, of organ and systemic infection, tumour formation, and tumour immune responses outside of the whole animal. The mouse is a very good model system as it closely resembles human physiology. More sentient systems, such as Drosophila or fish, have very different immune systems and lack defining parts of the human (and mouse) immune system.

Complex interaction of various host cells and -metabolites, and with the environmental cues and the microbiota are critical in the disease processes we study. There are no 'more reductionistic' model systems available that would reproduce the processes in human disease to a meaningful extent. We recognise that our mouse model systems have limitations and cannot reproduce all the conditions associated with human disease. However, mice have many similarities to humans, including in terms of their immune system and metabolism.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are monitored throughout all interventional experiments and we collect daily scores composed of a set of physical signs of illness such as piloerection (raised hair as a sign of pain or discomfort), hunched walk and mobility along with weight loss. The cut-off for these physical signs lies within the guidelines for mild, and in a few contexts, moderate severity, i.e. loss of pre-set percentage body weight being our main indicator, along with mobility (ability to feed and water). The scoring for piloerection etc. are also used as secondary indicators. My team are experienced in animal models and are trained to the high standards that I expect. The technicians that work in our holding facility and do the majority of the animal husbandry will also be trained by my team and will communicate abnormal behaviours in the mice early. At our establishment we have dedicated Named Animal Care and Welfare Officer who are impartial and can give advice / make decisions

on animals that lie outside of the normal adverse effects expected for the infections outlined within this project.

Potential refinements include increased monitoring if test animals show earlier clinical signs or weight loss. We will give wet mash food to animals that lose more weight quickly. Floor food will be given to animals that are to be infected to limit weight loss from the start of the infection.

Any evidence in pups not developing or litter losses will be discussed with NVS and appropriate actions taken. All animals will be given environmental enrichment and be socially housed to encourage natural behaviours and reduce stress. Males will be monitored for increased aggression and additional enrichment added to combat or appropriate splitting of fighting animals if needed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use guidance from the NC3Rs website and the Laboratory Animal Science Association (LASA) to ensure experiments are conducted appropriately. In particular we will follow the 'Guiding principles on good practice for Animal Welfare and Ethical Review Bodies'.

We will follow the PREPARE guidelines for planning experiments and will follow the ARRIVE guidelines reporting of results.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep informed on advances through the NC3R’s website, Norecopa website, our establishment website and newsletter. We will discuss any advances with the relevant people at our establishment and implement them accordingly.

108. Biobehavioural basis of the individual vulnerability to impulsive/compulsive spectrum 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

emotion, motivaiton, impulse control, addiction, individual vulnerability

Animal types	Life stages
Rats	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's the aim of this project?

The aim of this project is to understand the environmental, psychological and biological factors that contribute to individual vulnerability to develop the compulsive behaviours that represent the hallmark features of Impulsive/Compulsive Spectrum Disorders (ICSDs) such as Obsessive-Compulsive Disorder or drug addiction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 cost incurred by ICSDs to our society and the burden they put on our health care system reflect mainly the limitations of our current knowledge of both their pathophysiology and aetiology. This limited understanding thereby prevents the development of novel,

more effective preventive and therapeutic strategies.

It is therefore of paramount importance to better understand the biobehavioural basis of the individual vulnerability to develop compulsivity.

Thus, why only some individuals exposed to stress, experience distress or take addictive drugs recreationally lose control over their behaviour and go on to develop compulsivity remains a key question to be addressed that goes beyond the understanding of the neurobiological adaptations to distress, chronic stress or drug exposure. Indeed, since we are all subjected to these challenges it is pivotal to dissociate the psychological, neurobiological and environmental mechanisms that support adaptive coping strategies and impulse control from those which, in vulnerable individuals, aberrantly support the switch to compulsivity.

Additionally, this work will shed new light on our understanding of adaptive coping strategies which current and future generations will need to deploy in the face of new challenges, such as social media or globalisation.

What outputs do you think you will see at the end of this project?

This research will deliver new knowledge that will enhance our understanding of the biobehavioural basis of the vulnerability to develop Impulsive/Compulsive Spectrum Disorders (ICSDs) and identify new drug targets.

Outputs for all objectives are primarily behavioural and data collected through performance on specific procedures aiming to operationalise in the rat impulsive or compulsive behaviours or associated behavioural traits of vulnerability that have heuristic value with regards to the human situation.

Our understanding of the neural and cellular substrates of these behaviours will come from associated neurophysiological outputs such as neuronal activity patterns and circuit mapping in behaving or anaesthetised rats. Our understanding of the molecular mechanisms of these behaviours, within the identified neural circuits, will stem from investigations of the expression of specific molecules (such as metabolic products, mRNAs, proteins, small molecules acting as neuromodulators) within brain tissue assessed post mortem. Usually, molecular data are generated using the same procedures as for behavioural data, but animals are killed at a specific experimental time point whereas the investigation of neurophysiological and neuropharmacological processes in non-behaving animals is performed under terminal anaesthesia. Thus, for some of our objectives, the brains of the rats will be harvested for subsequent post-mortem analyses (using a wide array of contemporary neuroscience techniques, such as in situ hybridisation, RNAscope, immunohistochemistry, western blot, qPCR, next-generation omics, single-cell transcriptomics...).

Who or what will benefit from these outputs, and how?

The intended overall benefit of this research will be directed to Society as the intended increased understanding of the biobehavioural basis of ICSDs will have far reaching social and economic implications.

The first beneficiaries of this research will be the scientific and clinical communities. The publication in journals with Open Access, which, for basic research, remains the most effective route to communicate research findings and rapidly to gain international impact

and prominence, will only be the first step in a wider dissemination and communication strategy aiming to immediately increase our impact on the general public. Our goal is to help the general public realise that compulsive disorders, and especially addiction, are indeed psychiatric conditions that are not simply the behavioural manifestation of poor willpower, a view that has long contributed to a damaging prejudice towards those who suffer from these conditions.

The second beneficiaries would be the patients themselves as the output of this research will help improve the awareness of ICSDs as brain disorders, and facilitate their prevention and treatment by personalising medicines and other interventions, including at school, in those individuals deemed most at risk from developing maladaptive coping mechanism and/or harmful levels of drug use in the future. Such long-term benefits will depend on the efforts we continually make to provide to inform the general public and clinical policymakers.

We also intend to impact pharmaceutical company policy as they return to medications development for disorders such as addiction. We have therefore recently developed a jointed research project on drug addiction with a pharmaceutical company.

How will you look to maximise the outputs of this work?

We are fully committed to communicating our research as widely as possible to academic and scientific communities. The outcome of our research (may the results be positive or negative) will be published and disseminated to the scientific community in both high-impact scientific journals with Open Access and at national and international meetings and summer schools. Our results will be targeted for publication in peer-reviewed high impact journals (e.g., Science, Current Biology, Nature Neuroscience, Neuron, Biological Psychiatry, Neuropsychopharmacology, Journal of Neuroscience, Molecular Psychiatry). We have a strong track record in maximising the output of our work, with the systematic publication of negative results.

Manuscripts accepted for publication will be made open-access and archived in an institutional repository to ensure the widest possible accessibility and impact of our work, thus meeting the new HEFCE policy on peer-reviewed articles and conference proceedings. We will also continue to publish conceptual papers and commentaries which are often highly cited and increase the profile of our work and the field in general. This activity greatly increases the research profile of my group and enables the rapid communication of new findings to a wide scientific community.

Our results will be disseminated through presentations (plenary lectures, symposium lectures, poster presentations) at international and national conferences and summer schools.

We are fully committed to communicating our research as widely as possible to non-scientific communities. The publication in journals with Open Access will only be the first step in a wider dissemination and communication strategy aiming to immediately increase our impact on the general public. Our goal is to help the general public appreciate that compulsive disorders, and addiction especially, are psychiatric conditions and not simply the behavioural manifestation of a poor willpower, a view that has long contributed to a damaging prejudice towards those who suffer from the condition. Thus, we will rely on publicisation of our work by local and national media groups.

We will continue actively to participate in local and national public engagement events to

educate the public about our work, including teachers and students at schools and other educational institutions.

We will also use other means to increase our impact, especially through the powerful vehicle that represents arts.

We are fully committed to communicating our research as widely as possible to clinically active scientists and clinicians. We intend also to have an impact on approaches to treatment. Addiction theory and computational models have also evolved to incorporate notions of inhibitory control, actions and habits rather than earlier assumptions that the key to addiction is to be found exclusively in the dopamine reward pathway, which is instead the point of entry of addictive drugs to exert their rewarding effects.

We will investigate the translational potential of our findings in human subjects as a way to validate our discoveries in rats. The successful demonstration of cross-species convergence would enable targeted interventions to be developed based on candidate neural markers.

Species and numbers of animals expected to be used

- Rats: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The 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.
However, around 10% of those who take drugs eventually lose control over their intake and develop the compulsive relapsing pattern of drug seeking and taking that characterises addiction. In other words, we are not equally vulnerable to developing maladaptive coping strategies and/or drug addiction.

The environmental, psychological and biological factors contributing to this individual vulnerability to lose control over coping strategies or drug use, which results in Compulsive Disorders such as Obsessive Compulsive Disorder or drug addiction, remain poorly understood. This prevents the development of novel, more effective therapeutic or preventive strategies for these debilitating disorders that affect millions of individuals worldwide.

Thus, our research interests itself with the identification of the environmental, psychological, neural and cellular factors that contribute to the individual vulnerability to develop Impulsive/compulsive Spectrum Disorders (ICSDs) across several nosological boundaries.

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 the investigation of 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. The study of the environmental, psychological and biological basis of the vulnerability to develop as debilitating a psychiatric condition as OCD or drug addiction requires the establishment of these conditions in the animal model. However, our experimental approach has been refined so that the overall suffering induced by the development of these compulsive behaviours

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.

For this, we have a dedicated optimisation and maintenance licence the purpose of which is to optimise and refine each of the procedures that will be used on relatively large cohorts of animals under the present one. 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.

We take the welfare of the animals very seriously. Most of our animals are exposed to 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 and handled 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 Named Veterinary Surgeons 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 killed to preventsuffering.

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. Each of our experiments is designed on the back of a robust statistical approach that relies on state-of-the-art statistical tests and models.

Typically, what will be done to an animal used in your project?

Rats used in this project (including GA animals with no adverse phenotype) will be exposed to several sets of behavioural and experimental procedures the cumulative severity of which never extends beyond moderate severity.

The various procedures rats will be exposed to aim at measuring their individual performance in tasks that operationalise, for instance, attention, behavioural flexibility, impulse control, anxiety trait, decision making, boredom susceptibility, interest in appetitive outcomes and sensitivity to negative ones (such as mild electric foot shocks) as well as goal-directed or habitual instrumental responding. Testing in these tasks, as well as coping-related tasks sometimes requires rats to be exposed to food restriction and single housing conditions for the entire duration of an experiment (up to 12 months).

Rats (no more than 30%) will be exposed to procedures that enable the assessment of adaptive, or maladaptive (compulsive) coping strategies in the face of distress; these procedures require that rats are food-restricted (between 80% and 85% of their theoretical free-feeding weight) and singly-housed for a period of at least 15 days and up to 12 months overall.

Our research also focuses on the contribution of interoceptive mechanisms to the vulnerability to develop ICSDs. For this we use a drug discrimination task that requires systemic administrations of a drug over multiple daily sessions. Thus, no more than 40% of the rats may receive multiple systemic administrations of substances that trigger specific interoceptive states or dependence. These may also be administered with substances that counteract these dependent states.

In the case of drug self-administration, rats (no more than 50% overall) are given the opportunity freely instrumentally to respond for a bolus of cocaine, heroin or other addictive drugs delivered directly into their bloodstream through an indwelling catheter connected to a syringe. Most rats maintain control over their drug intake, as humans do, but some, those which underlying psychology and neurobiology we seek to understand, eventually develop drug seeking habits that persist despite adverse consequences (such as exposure to response-produced, hence escapable, mild electric foot shocks), the hallmark of the compulsive nature of addiction in humans. These shocks are always avoidable and their maximum intensity kept very low. There is no alternative to such procedure to measure the persistence of a behaviour despite negative consequences that characterises compulsive disorders.

Rats (no more than 40% overall) may be exposed to several stressors such as maternal separation, social defeat and/or mild unpredictable stress to measure the influence of stressful events on their subsequent resilience or vulnerability to develop impulsive/compulsive spectrum disorders (ICSDs).

Alone, or in conjunction with any combination of the previously described procedures rats (up to 50%) may be subjected to intracerebral manipulations either while behaving or under terminal anaesthesia. Thus, they will receive up to 2 intracranial procedures, prior to any additional procedure performed under terminal anaesthesia.

Rats lay also be implanted with subcutaneous minipumps or slow-release formulations in order chronically to influence a central or peripheral system and/or trigger dependence. Half the animals that will have developed dependence will then experience precipitated withdrawal.

The investigation of endophenotypes of vulnerability requires blood samples to be collected from peripheral vessels from the animals (no more than 30% overall). No more than 10% of total blood volume will be collected in any 24hr period and not more than 15% in any 28-day period.

Rats (no more than 10% overall) may experience experimentally-induced Parkinson's disease because the study of the individual vulnerability to develop Impulse control disorders in Parkinson’s Disease requires a Parkinson’s Disease state is induced in behaving rats.

The investigation of specific cellular mechanisms requires the decapitation of the rats in a conscious state.

What are the expected impacts and/or adverse effects for the animals during your project?

For the majority of our animals on this project (75%), we anticipate no more than transient discomfort and no lasting harm.

When rats undergo surgical procedures, they 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 toenails and toes can inflict damage to their paws. While there is no alternative to these side effects of chronic exposure to heroin, these adverse effects are minimized by early end points.

The study of the biobehavioural basis of drug addiction that intends to have a real impact on the life of the millions of individuals who suffer from this debilitating psychiatric disorder requires the identification of compulsive, maladaptive behaviours in a subset of animals exposed to the drug for long periods of time. As a matter of fact, over the past two decades, all the behavioural, psychological and biological mechanisms that have been identified in the rat to underlie this vulnerability to develop compulsive drug seeking and taking using our methodology have been subsequently demonstrated I humans, thereby demonstrating the clear translational value of our multidimensional and heuristic approach.

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). There is no alternative to the induction of dependence and withdrawal to study the cellular and molecular basis of these phenomena and their contribution to compulsive behaviour.

When rats are encouraged to develop coping strategies in the face of distress, some (no more than 30%) lose control over their coping strategy and progressively develop

behavioural manifestations that are similar to those shown by individuals suffering from Obsessive/Compulsive Disorder. Thus, these rats show excessive ritualised behaviours such as grooming and adjunctive drinking behaviour. These behavioural are not harmful to the animal.

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 animal type)?

Protocol 1: Moderate, 100%

Protocol 2: Moderate, 100%

Protocol 3: Moderate, 100%

Protocol 4: Moderate, 100%

Protocol 5: Moderate, 100%

Protocol 6: Moderate, 100%

Protocol 7: Moderate, 100%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not yet possible to achieve our objectives without using animals, and, especially, rats. Our research investigates the individual vulnerability to develop impulsive/compulsive spectrum disorders, which results 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 procedures carried out under this PPL will have systematically be optimised, including using our optimisation Licence.

Which non-animal alternatives did you consider for use in this project?

We use alternative strategies some of which involve humans, some are computer-based models and some use Artificial Intelligence. For instance, having shown in rats that a high impulsivity trait predicts and exacerbated vulnerability to develop early-onset Parkinson's Disease, we have established sibling studies combined with brain imaging analyses in human populations to further investigate the biological and environmental basis of such vulnerability. We have shown that the siblings of early-onset PD patients were more impulsive than those of late-onset PD patients, thereby confirming in humans the striking observation originally made in rats. Similarly, having shown in rats that the development of maladaptive habits results in an exacerbated vulnerability to relapse mediated by negative urgency, we have designed longitudinal studies in humans combined with EEGs to further the neural correlates of the influence of negative urgency on craving and relapse. 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) in order to identify responses to drug exposure at the cellular level that do not require a behaving organism.

Why were they not suitable?

As our objectives, which will be achieved through the use of a complementary project licence, 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 across the lifespan that is the focus of this research.

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?

The number of animals required for these experiments is based upon the historical usage of animals in our laboratory. Overall, with a requirement of 48 to 96 rats per experiment, we have used ~6000 rats in the last five years in spite of the COVID pandemic. In the next five years we will therefore use a maximum number of 6400 rats. These numbers reflect the absolute maximum range of the estimated numbers for the next five years.

Provided our research is mostly based on inter-individual differences, when the distribution of the population allows for an objective criterion to select specific groups such as a bimodal distribution, the group size is dependent upon the distribution. In the other cases, the groups used to perform between- subject analyses will be selected in the upper and lower quartiles of the population.

In order to maximise the differences between the experimental groups, we will, therefore, carry out experiments with cohorts of 24 n animals, with 1<n<4 for each experiment. We do not foresee the use of cohorts of more than 48 rats for any of our experiments.

Our group sizes (n= 5-12) are based both on power statistics and reference to the established scientific literature that are necessary to obtain statistically and biologically meaningful data with an initial target of alpha = 0.01. By applying this strategy, we are very confident that results will be statistically relevant even if, especially with protracted self-administration experiments, we have to kill animals during the experiment.

For experiments for which we can make no predictions about the size of the behavioural effect a manipulation can cause or how a behavioural difference relates in terms of neurophysiological or cellular processes, these experiments are conducted over several runs, and the early runs are used to calculate effect sizes and to perform power analysis to inform the final group sizes.

If we typically anticipate n = 5-12 animals per group, our between-subject design analyses will always be computed along with dimensional (correlation, regressions, factorial regressions, PCA, cluster analyses) analyses in order to make the maximum use of each individual within a design aiming at emphasising inter-individual differences.

We have recently implemented a new strategy whereby each study that requires a final cohort of 48 rats is often (wherever appropriate) ran in two independent experiments. Thus, not only do we ensure internal reproducibility of the effects observed between the two runs, but provided we reach a statistically and biologically meaningful result at the end fo the first experiment we are in a position not to run the second experiment and spare half the number of animals initially planned.

Wherever possible, we use within-subjects experimental designs (e.g., testing the effects of different drug doses on a specific ongoing behaviour in a Latin-square design) as this increases statistical power and reduces the numbers of animals required.

Where between-subjects comparisons have to be made (e.g., where each animal’s behaviour can only be manipulated once without compromising the interpretation of subsequent data) there are a minimum of two experimental groups (one control group and one experimental group), and sometimes additional controls (e.g., if we need to

demonstrate that a drug given systemically is acting in the brain to influence behaviour, then a similar drug that does not cross the blood-brain barrier may be given to another control group). If the vehicle for the peripherally-acting drug and the centrally-acting drug differ, then another vehicle group may be included for the peripherally-acting drug).

For all experiments, we use the minimum number of control groups that we can in order for the experimental data to be interpretable. Nevertheless, we will make sure our group size is big enough to start with in order to avoid having to replicate an experiment in which only specific individuals (i.e., upper and lower quartile) are taken into considerations for between-subject analyses, which would run counter to the principles of the 3R's. We are indeed familiar with the PREPARE and ARRIVE guidelines (https://proecopa.no/prepare; https://arriveguidelines.org) and will ensure all our experiments are designed in adherence to these guidelines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are committed to, and have succeeded in, using the fewest animals required for statistically and biologically meaningful results. Recent publications in high impact journals, the data of which were acquired under the current PPL, report highly novel and impactful results with relatively low sample, but big effect (e.g., partial eta squared values) sizes.

Our approach, based on inter-individual differences, inherently makes uses, and capitalizes on individual variability, rather than trying to control for it, alongside a highly standardized behavioural neuroscience methodology that dramatically minimizes external sources of variability that contribute to decreasing the power of our experiments.

We will continue to design our experiments using the minimum number of animals deploying a range of strategies that we have implemented over the years and were previously validated by a biostatistician. The key principles on which our statistical design relies are:

Aiming for an overall statistical level of significance of $p < 0.01$ and large effect size (partial eta squared values (>0.14) which we report systematically in our publications) between individual groups, i.e., animals showing vulnerability, and those showing resilience to, neuropsychiatric disorders, generally selected in the upper and lower quartiles of the population or between experimental conditions, i.e., chemogenetic inhibition of a specific neuronal ensemble vs non inhibited control.

Combining between (or within when possible) subject design with dimensional analyses in order to strengthen the power of the overall conclusions. We have been very successful in applying various descriptive analyses including cluster analyses, factorial analysis or Principal Component Analysis with either simple, multiple or factorial regression analyses to draw firm conclusions from relatively small samples.

Optimising animal use through conducting 'pilot' experiments under our maintenance and optimisation licence to verify that brain manipulations or biological measurement are producing the intended effects at a neural level, and to calculate effect sizes (and statistical power) to identify how many animals are required for analysis.

Using within-subjects designs whenever possible, as these have greater statistical power than between-subjects designs and require fewer animals.

Blinding the experimenter to the behavioural data for the post-mortem histological verifications or interrogations of the neural, cellular and molecular substrates or correlates of the psychological and behavioural mechanisms under investigation.

Over the past year, we have developed new machine-learning-assisted classifiers to characterise rats that are resilient to, or at risk of developing addiction irrespective of the cohort to which they belong.
This new tool (publication under preparation) will be deployed in all our experiments to further reduce, wherever possible, the size of the cohorts we initially train to study identify those individuals that eventually develop ICSDs.

We have much expertise in experimental design and statistics, ranging from parametric to non- parametric analyses, sophisticated analyses of variance and covariance, and associated post-hoc tests, dimensional analyses, such as multiple and factorial regressions, as well as data mining such as k-means clustering, principal component analysis and distribution fitting. However, if further advice is needed, we will consult statisticians.

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 implemented a standardized approach of training cohorts of 48 rats at the same time, allowing us to obtain at least 5 to 10 rats (depending on the nature of the compulsive behaviour under investigation) with extreme behaviours from a cohort with a very similar history, may it be related to handling, behavioural training, housing conditions or surgery and peri-operative care.

To this aim, we have developed highly standardized procedures and high throughput surgical techniques under aseptic conditions. The latter is also very important for ensuring that catheter/cannulae/probes life expectancy of the animals undergoing surgery first is not reduced by the number of days of surgery spent on subsequent animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

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 are subject to invasive recordings or manipulations of the brain while performing these tasks.

We are fully committed to minimising 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 to 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. Alternatives, such as genetically engineered SAPAP mouse, display profound adverse phenotypes such as excessive self-grooming-induced skin lesions.

When rats are food restricted, they often need to be singly 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 the 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. Indeed, we have 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, they 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. In addition, we demonstrated that the exacerbation of the vulnerability to develop compulsive drug use in rats by environmental enrichment generalised to alcohol since rats raised in an enriched environment are more likely than rats raised in a standard environment to relapse to alcohol drinking after abstinence and to compulsively do so, in that they would persist in drinking alcohol at relapse even if it is adulterated by the bitter tastant quinine.

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. There are no alternatives to the use of these procedures, but our constant refinement has helped us achieve an attrition rate of almost 0% by 80 days of daily training while the average worldwide is over 5% by two weeks, with only two other laboratories in the world reporting

chronic self-administration experiments lasting more than 6 weeks, but with an attrition rate of over 15%.

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.

In order to determine the role of stress during neurodevelopment and or adolescence/adulthood, rats will be exposed to maternal separation stress and/or one of two types of stress during adolescence and/or adulthood. Our stress procedures have all been refined and are far superior to alternatives that, for most of them, require exposure to inescapable shocks, restraint and/or forced swimming.

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 a 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 animals that are less sentient?

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.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Any new procedure, or combination of procedures, used under this project licence will systematically be previously optimised using my dedicated optimisation licence.

All our current procedures are constantly being refined.
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 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. Similarly, we have shown that environmental enrichment exacerbates the vulnerability to relapse to alcohol drinking and to persist in doing so despite the adulteration of acohol by a bitter tastant such as quinine.

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 unique procedures undertaken under this licence will have previously been optimised and refined, either as part of our ongoing refinement strategy or using my optimisation licence. Thus, rats on this licence undergo neural manipulations (brain

surgery) and/or implantation of intravenous catheters for long-term self-administration of drugs of abuse that will have been systematically optimised. Indeed, 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 effort 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 was 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 you follow to ensure experiments are conducted in the most refined way?

Our research is constantly guided by, and adheres to the Laboratory Animal Science Association (LASA), the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and the ARRIVE (Animal Research: Reporting In Vivo Experiments) Guidelines. Not only do we follow the LASA guiding principles of aseptic surgery (http://www.lasa.co.uk/wp-content/uploads/2017/04/ Aseptic-surgery-final.pdf), but we have furthered these principles wherever possible as part of our constant refinement strategy, especially in the case of intra-jugular catheter implantation procedures.

We will receive direct updates on best practice from the N3CRs as I have subscribed to their mailing list.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the 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.

109. Development of peptide drug conjugates for cancer therapy

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph b

Key words

cancer, pancreatic, glioblastoma, therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

All the cells that make up a human have an outer layer (the cell membrane), a little like a very strong soap bubble, that is made up of a mixture of different building blocks (lipids). Scientists have collected a lot of data over the last 10 years that indicate that the outer layers of cells in cancer tumours have different building blocks from healthy cells. The aim of this project is to see if it is possible to make cancer drugs which can stick only to these unusual building blocks and not to the building blocks seen in normal healthy cells. If this is possible then we can use them to target cancer therapies only to a patient’s tumour so that they don’t suffer from serious side effects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a very common illness: one in every two people will suffer from it at some point in their lives. Even though many treatments have been discovered and some types of

tumours are now treatable people still suffer from unpleasant side effects. In addition, there are many types of cancer which are still as serious now as they were more than 40 years ago as they have proven difficult to treat well.
Pancreatic cancer is an example of one of these. Only 7% of people who are diagnosed with pancreatic cancer are still alive after 5 years.

From information we have gathered so far, we think that potential cancer drugs which target the differences in the outer layer of tumour cells could work very well for most cancers but particularly for pancreatic cancer and for cancers which have come back after being treated with currently available medicines.

What outputs do you think you will see at the end of this project?

The work we have planned for this project is designed to answer the following questions:

Do our potential cancer drugs only go to tumours and not to healthy parts of the body?

If they only go to tumours, do they shrink or get rid of the tumour?

Are they likely to be safe enough to be tested in human volunteers?

This information will help us decide if it is worth developing these molecules further towards becoming a drug that can be available to people suffering from cancer.

Who or what will benefit from these outputs, and how?

In the short term (1 - 2 years), our work will help scientists decide if this is a way of treating cancer that deserves further investigation. This will be a benefit to us as it will allow us to decide whether to focus time and money here or to investigate other potential treatments. In a medium term (3-5 years) we will be able to publish our results, either negative or positive, to allow other researchers to benefit from the
data generated which may lead to new ideas but also prevent others repeating work unnecessarily. These results will also form part of the information which helps scientists decide if the molecules are safe and effective enough to be tested in volunteers with cancer, giving patients early access to new medicines. In the long term (6 - 10 years), after further development and trials in humans, positive results in these studies could be developed into a new way of treating cancer which will benefit patients with difficult to treat tumours or cancer that has come back after treatment.

How will you look to maximise the outputs of this work?

We are collaborating on this work with two academic groups in the UK and will be sharing our results through these collaborations as well as by publication of results. We are also seconding researchers to one of these institutions so that they can share the methods and techniques we are developing.

If the project is successful, we will license the project to a drug developer to ensure the research has a high chance of resulting in improved medicines for cancer patients.

Species and numbers of animals expected to be used

- Mice: 870

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have already tested our potential medicine (compounds) on cancer cells in the laboratory and have found that the compounds attach to cancer cells, but not to normal cells and do not appear to damage normal cells. We now need to test the compounds in a way that is more similar to treating a tumour in a human patient. The options for this are to use a laboratory animal such as a zebra fish or a mouse. We have chosen to do this study in adult mice as previous work in the scientific community has used this model. This means we have a higher confidence that we will generate useful information and that we can compare our results to other scientists' work to make sure that we understand the results.

Typically, what will be done to an animal used in your project?

Typically, the mouse will be injected with some tumour cells just under the skin on its side. As this is expected to cause discomfort the mouse will be given anaesthetic. After about one to three weeks the tumour will have grown to approximately 5 to 12 mm in diameter. At this point a solution of the compound will be injected into the mouse. The mice will then be humanely killed after 0, 30 and 60 minutes (or similar). The tumour and other parts of the mouse will be dissected and examined to see if the test drug is going to the tumour. Alternatively, the mice will be monitored for two to three weeks after having the drug to see if the tumour stops growing, gets smaller or disappears. At the end of this period, the mice will be humanely killed and the tumour and tissues analysed to look at the effect of the drug.

What are the expected impacts and/or adverse effects for the animals during your project?

When the mice are injected either with tumour cells or the compound we are testing, they will experience brief discomfort similar to that which a person might feel when getting a vaccination. As part of the study, the mice will grow tumours just under the skin. We do not expect these to grow large enough to cause the mouse significant illness. Some mice will also be treated with cancer medicines which may cause them to feel unwell in a similar way to a human cancer patient. At the end of the study each mouse will be humanely killed in a way designed to cause the minimum of stress or discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?
Most of the mice (estimated 75%) will experience mild procedures (causing only brief discomfort like a vaccination would for a person). The remaining 25% of mice may experience moderate severity, if for example their tumour grows very quickly, or they feel ill from the chemotherapy given to them.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A key aim of the project is to determine whether the compounds concentrate in tumours. To be able to investigate this it is important to inject the compounds into a living animal so that we can follow what happens to the compound as it travels around the body. We will then be able to see if the compounds go to the tumour and do not go to the healthy parts of the body.

In addition, we want to check that when the compounds go to the tumour, the tumour is reduced in size. Cancer tumours are very complicated, and it is difficult to accurately reproduce them outside of a living animal.

Which non-animal alternatives did you consider for use in this project?

We have reviewed the published scientific literature to identify potential replacements for animals. We have considered using tumour cells, tumours grown in the laboratory and artificial “bubbles” made with the same building blocks that appear in cancer cell walls (liposomes). As a result, we have used tumour cells and artificial “bubbles” (liposomes) in our own laboratories to conduct experiments before using animals.

Why were they not suitable?

Although laboratory models can give an indication of the behaviour of a compound in an animal or a human, they do not have the full complexity of a living organism and are not accurate representations of a tumour or of normal healthy tissue. For example, tumour cells grown in the laboratory do not have a blood supply and models of healthy cells always have changes which allow them to survive in the laboratory but do not make them a good model of normal cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

We have calculated how many animals are needed for each experiment to make the result reliable and how many experiments we will need to conduct over the next 5 years to achieve the objectives of our research and so fully test whether the drugs we are investigating could help cancer patients.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3Rs Experimental Design Assistant and publications from specialist statisticians working in cancer to develop experimental designs which maximise the information obtained while minimising the number of animals used. This includes studies designed to minimise the variation caused by, for example, differences in cage environment and also designs to test multiple factors in one experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will be getting the mice from a large breeder so that we are only using the animals we need to carry out the study and therefore minimising inefficient breeding which produces too many animals. Prior to starting these studies, we have carried out small pilot studies to identify important factors such as rate of growth of the tumour, how much of the compound to give the mice to see an effect and to check we are able to analyse the results properly.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using mice that have been specially bred to be able to carry tumours made from human cells without it causing them to reject the non-mouse cells. The cells are injected just under the skin. This means that the mouse is not growing tumours inside its organs or bones that could cause it suffering but carries the tumour under the skin on its side. This model causes the least amount of suffering to the mouse but gives useful data as the tumour is made of human cells.

Why can’t you use animals that are less sentient?

We have considered using flies or fish, but it is difficult to implant human tumours into these animals. The adult mouse is a mammal with a similar biology to humans and makes a more accurate model and will generate more meaningful data.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will make sure the mice are given time to get used to being handled so that injections and blood samples are as stress free as possible. Any animals that do not adapt to handling will be removed from the study. Where pain is likely to be experienced, we will use anaesthetic. The growth of the tumour will be monitored so that it does not grow big enough to cause the animal distress. We will also monitor the weight and condition of the animals and any showing signs of ill health will be humanely killed. Animals will be housed

in small groups in cages with comfortable bedding and nesting materials as well as shelters and toys so that they are comfortable and occupied when not being studied.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow “ARRIVE” and “PREPARE” guidelines which have been written to assist scientist to conduct good quality studies and to share the results. We will use the LASA “Administration of Substances Guidelines” and the “Code of Practice for the Housing and Care of Animals Bred, Supplied and Used for Scientific Purposes” to ensure the animals are well cared for. In addition, we are following the recommendations set down in “Guidelines for the welfare and use of animals in cancer research” published by P Workman in the British Journal of Cancer in 2010.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will review the scientific literature every three months to identify any new work which we could use to reduce or refine the study we are carrying out. In addition, we will be advised by our local animal welfare committee (AWERB), training officer (NTCO), named information office (NIO) and vet (NVS) and review the newsletters originating from organisations such as NC3Rs.

110. Haematopoietic stem cell mechanisms and 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

Haematopoietic stem cells, Blood cell production, Haematological diseases, Aging, Cell and gene therapy

Animal types	Life stages
Mice	adult, embryo, aged, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to discover novel biological and genetic regulators of blood-forming haematopoietic stem cells, define how genetic mutations alter haematopoietic stem cell activity, and identify new approaches to use haematopoietic stem cells in cell and gene therapies. The ultimate goal is to develop new stem cell-based therapeutic strategies for haematological diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work has the potential to improve the treatment options and even cure (or prevent) many haematological diseases. This includes a range of haematological malignancies and

age-related diseases (e.g., myelodysplastic syndrome, acute myeloid leukaemia) as well as inherited blood diseases (e.g., sickle cell disease, immunodeficiencies, Fanconi anaemia). Together, haematological diseases represent a major disease burden worldwide, and there are few safe curative treatment options available for most blood diseases.

What outputs do you think you will see at the end of this project?

The outputs of this project are expected be:

Discover new biological and genetic regulators of blood stem cells self-renewal and multipotency

Establish new therapeutic approaches to treat and cure haematological diseases

Contribute to peer-reviewed publications

Generate pre-clinical data to support the initiation of clinical trials

Who or what will benefit from these outputs, and how?

Beneficiaries of these outputs will be:

Biomedical researchers. Through peer-reviewed publication and dissemination of our findings from this project, we hope to help other researchers (interested in stem cell biology, haematological diseases, and/or cell and gene therapies) make further discoveries and technical advances in this field.

Patients suffering from haematological disease. We hope that this work will ultimately benefit patients through provide new and safer clinical treatment options.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised by:

Peer-review publication. We will publish our findings and approaches in peer-reviewed publications in order to share our discoveries with the biomedical research community and public. This will include unsuccessful approaches to help other research avoid repeating our work.

Presentation at scientific meetings. To further disseminate our findings within the biomedical community, we will present our work at scientific meetings.

Collaborations. We will actively collaborate with other academic biomedical researchers to share knowledge and expertise from our project. Synergistic collaborations can be powerful way to maximise research productivity. Where appropriate, we will also collaborate with industry.

Intellectual property protection. Where appropriate, we will protect intellectual property from this project through our technology transfer office. This will help to maximise the benefit of the research and allow for external investment to fully develop new therapeutic approaches while disclosing the intellectual property with the public.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are one of the least sentient mammalian species. As we are investigating the mammalian haematopoietic and immune systems, simpler animal species cannot be used. Additionally, decades of scientific research and development mean that numerous mouse experimental protocols (cell type markers, culture protocols, genetic engineering methods, etc.) are readily available and we can maximise the welfare of these animals in our experiments. To avoid confounding effects of puberty, we use 8-12 week old mice as "young adults". For experiments where we are investigating aging, we use ~18-month old mice as "aged adults". Use of these timepoints also allow us to compare our results with historical datasets, which have used these ages.

Typically, what will be done to an animal used in your project?

Typical animal experiments used in this project will be:

Breeding and maintenance of mice, including breeding of genetically altered mice. Most of these mice will be killed for harvesting haematopoietic cells. Some mice will be used for breeding and then killed, or used as recipient in transplantation assays (see below). Transplantation assays. This involves injecting donor haematopoietic cells into a recipient mouse. In some cases, the recipient mice will be pre-conditioned using radiation to help support donor cell engraftment. Donor cells are tracked over time through periodic bleeding of the recipient mice. At the endpoint, recipient mice are killed to allow for bone marrow and organ analysis. Transplantation assays can be performed using mouse or human cells.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts and/or adverse effects for the animals on this project are:

We will be using genetically altered animals as models of human diseases, e.g., Fanconi anaemia and sickle cell disease. Complications from these diseases are expected in a small number of cases and can lead to death; these mice will be killed.

The radiation used to pre-condition mice in transplantation assays causes radiation damage. In most cases, this causes a transient radiation sickness and weight loss at 1-2 weeks post-treatment, from which the mice recover. On very rare occasions, this can cause death. Mice that do not recover will be killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 35% subthreshold, 60% mild, 5% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project needs to use animals because:

Haematopoiesis (blood formation) is a complex cellular process that requires numerous local and systemic signals from the bone marrow microenvironment and other tissues, that cannot be accurately modelled in vitro or in silico.

The functional potential of haematopoietic stem cells can only be determined by assessing their activity in transplantation assays. Haematopoietic stem cells currently cannot be generated through in vitro methods.

Which non-animal alternatives did you consider for use in this project?

We considered in vitro assays as alternatives for this project including in vitro use of haematopoietic cell lines and in vitro differentiation of pluripotent stem cells into haematopoietic cells. We also considered ex vivo culture of primary haematopoietic cells and in vitro colony forming assays to assess differentiation potential. All methods will be used where appropriate to reduce the use of animals.
However, none of these alternative methods can fully replace the use of animals.

Why were they not suitable?

In vitro haematopoietic cell lines are not suitable because they are transformed (cancer) cell lines that do not recapitulate the normal activities of primary haematopoietic stem cells. For example, transformed cell lines are usually genetically abnormal and fail to differentiate.

In vitro pluripotent stem cell differentiation into haematopoietic cells is not suitable because these methods cannot generate functional haematopoietic stem cells. Additionally, these methods generate embryonic-like haematopoietic cells that are distinct from adult haematopoietic cells.

Ex vivo culture of primary haematopoietic cells and in vitro colony forming assays will be used where possible. However, these methods are not suitable to evaluate the full functional activity of haematopoietic stem cells, which are defined by their ability to reconstitute the entire blood and 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?

Experimental cohort sizes will be estimated based on previous experience with these experiments. Breeding calculations have been utilised to estimate the number of animals required to produce cohorts of relevant genotypes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This programme will take advance of recent developments in ex vivo expansion culture technology for mouse haematopoietic stem cells. This new technology allows us to expand haematopoietic stem cells ~900-fold and therefore substantially reduces the number of donor animals required for HSC isolation. The NC3R's Experimental Design Assistant was used during the experimental design of this project.

We will comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines) when publishing the work coming from this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Measures to optimise the number of animals planned to be used in the project include:

For commonly used inbred mouse strains (such as C57Bl6/J and its CD45.1 allotypic derivative), we will purchase these mice from centralized breeding colonies. These colonies are maintained at scale and regularly re-started from frozen stock to minimize genetic drift and bottlenecks, thereby maximizing the reproducibility of results over time, reducing the need for repeating experiments.

To minimize the number of animals used, breeding strategies will be optimized to generate as few surplus animals as possible. This will be accomplished by designing crosses that generate experimental and control animals in equal proportions, and as few mice with genotypes that are not useful as is possible.

Cryopreservation will be used where possible to store transgenic mouse colonies without wastage.

When performing irradiation, we will split the dose over two sessions to reduce radiation toxicities.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Inbred wild-type and genetically altered mice will be used in this project. Decades of scientific research and development has established the mouse as the most suitable model for studying haematopoiesis and key procedures such as bone marrow transplantation and genetic modification have been most refined in mouse as compared to other species.

Breeding and maintenance of genetically altered mice: We use standard breeding techniques to maintain genetically altered mice for investigating the genetic regulation of haematopoiesis.
Conditional mutagenesis will be used wherever possible to restrict phenotypes to the relevant cell types and tissues, ensuring that any suffering is minimized.

Bone marrow transplantation assays: Most refined methods available will the used, including use of split dose radiation and use of individually ventilated cages. Additionally, where feasible for the experimental goals, we will perform transplantation assays without the use of irradiation (the most toxic step of the procedure), e.g., by using antibody-mediated conditioning or performing transplantation in the non-conditioned setting.

Why can’t you use animals that are less sentient?

Mice are one of the least sentient mammalian species. As we are investigating the mammalian haematopoietic and immune systems, simpler animal species cannot be used. Additionally, decades of scientific research and development mean that numerous mouse experimental protocols (cell type markers, culture protocols, genetic engineering methods, etc.) are readily available and we can maximise the welfare of these animals in our experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To refine the procedures to minimise the welfare costs for the animals in the breeding and maintenance of genetically altered animals, we will:

Closely monitor genetically altered mice for signs of disease presentation or pain. Mice will be killed where pain cannot be managed. Mice will be weighed regularly to detect weight loss.

Where possible, minimise breeding to reduce animal wastage.

To refine the procedures to minimise the welfare costs for the animals in bone marrow transplantation assays, we will:

Closely monitor recipient mice following radiation for signs of pain and distress. Mice will be killed where pain cannot be managed. Mice will be weighed regularly to detect weight loss.

Where possible, avoid use of radiation. This may be through the development and use of antibody- mediated conditioning. In certain cases, we will perform non-conditioned transplantation assays.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published best practice guidance available here: www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk. We will also regularly consult with our Named Information Officer, Named Veterinary Surgeon, and Named Animal Care and Welfare Officer to ensure our best practice guidance remains up to date.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs through:

Attending internal 3R meetings

Regularly read the latest literature

Regularly check up on the NC3Rs website

Regularly interact with the NC3R's regional manager and our Named Information Officer

Where relevant advances in the field occur, we will seek to effectively implement them during the project through revising our approved animal protocols. New techniques will be initially piloted to compare it with the establish technique. Where the new technique successfully refines our protocol, these will be implemented.

111. Parasite culturing in farm animals and farm birds, excluding coccidia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Parasite culture, Liver fluke, Parasitic nematode, Ectoparasites, Protozoa

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult
Goats	adult, juvenile
Pigs	adult, juvenile
Domestic fowl (Gallus gallus domesticus)	adult, juvenile
Domestic poultry	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to provide a service to commercial clients (in addition to our ‘in-house’ research groups) for the supply of parasites for use as controls in veterinary diagnostic testing, quality assurance, and research projects requiring such materials.

In order to ensure the parasite production complies with ASPA, we will:

Determine whether the parasites required are to be used for a qualifying purpose;

Determine whether the type and/or quality specification the parasites requires the use of live animals or whether parasites could be supplied from post mortem or from archived material;

Supply timely, high quality samples of parasites that meet the quality specifications for the work;

Refine and/or validate existing/new animal models/techniques in support of 3Rs initiatives and the purpose of this project.

This will be achieved using a client request form, the information provided being first reviewed by the PPL Holder for acceptability and conformance with a pre-agreed list with the AWERB, to help ensure compliance with the terms of the licence and to ASPA. Specifically, the client request form will identify the particular background, objectives and potential benefit of each request. The purpose for which the service is provided will be for a permissible purpose under ASPA and is specified in the project licence. An example of the Request form template has been uploaded as an attached document in ASPeL and includes the questions that will be asked of clients to inform our decisions about the suitability of their request.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Organisations conducting research on parasites can access a source of parasites with known origin and characterisation. The parasites cultured are of high quality, pure cultures and produced with high welfare standards.

What outputs do you think you will see at the end of this project?

Financial income for the company which will enable us to carry out further contract research projects involving parasites. We contribute to fundamental research which is used at world class institutes via the publication of scientific papers. We contribute to the understanding and control of liver fluke and produce material for use by other external customers.

Who or what will benefit from these outputs, and how?

We will benefit through the income generated by the use of cultured parasites in internal research projects and also by selling cultures to external customers. Research institutes, commercial businesses, farmers and animals via new products will all benefit from culturing work being carried out.

How will you look to maximise the outputs of this work?

We collaborate with academic organisations, publish scientific papers, attend relevant conferences and we play an active role in creating guidelines in this area of research.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 200
- Other birds: No answer provided

- Cattle: 40
- Sheep: 250
- Pigs: 15

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

No in vitro substitute for the host species currently exist to successfully culture the desired pathogens for in house use and external consumer use. We normally use young, weaned animals as they are the most naive to the parasite infection but produce the maximum yield with fewer adverse effects.

Typically, what will be done to an animal used in your project?

Infected orally, in feed, water or using a gavage. Infected via the skin for ectoparasites and the nematode Bunostomum in cattle. The host animals will be maintained to allow parasite development. The parasites will normally be recovered either at post mortem, via skin scrape or brush or from the faeces.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of our helminth experimental models are well developed and aim to allow the animals to remain clinically and behaviourally normal. When using a new parasite a new experimental model may be developed and we may encounter effects within the mild category. The duration of these effects could be several weeks meaning the severity category could increase to moderate.

Likely adverse effects and likely incidence:

The levels of infection/infestation chosen should not cause clinical disease of beyond mild but the duration ofinfection may increase severity to moderate.

Withdrawal of feed could result in stress to individual animals.

As far as possible, ectoparasite infestations will be maintained within the mild category, but work particularly with mites and blow flies, despite being carefully controlled, may be moderate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild and moderate. 40% mild. 60% moderate (see full breakdown in protocols). The maximum expected severity is moderate with any animals that reach this threshold being treated or euthanased as appropriate before they reach the severe threshold.

Animal types	Est. numbers (mild)	Est. numbers (mod)	Life stages
cattle	16	24	Adult and Juvenile
sheep	100	150	Adult and Juvenile
pigs	6	9	Adult and Juvenile
birds	230	345	Adult, Neonate, Juvenile

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not entirely possible to raise the parasite species outside of its host organism. We continue to look at NC3Rs website and relevant literature to assess if any non-animal alternatives are available.

At present there is no in vitro substitute for host species. We normally use young weaned animals as they are the most naive to the parasites but produce maximum yield with less adverse effects.

Which non-animal alternatives did you consider for use in this project?

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?

We use a combination of tools to estimate the numbers of animals needed. This normally includes the NC3Rs Experimental Design Assistant, VICH and WAAVP guidelines as well as consulting guidelines set by regulatory bodies and any relevant literature to ensure that the minimum number of animals is used.

Peer-reviewed journals and advice from external peers together with in-house experience and historical data over a number of years is used to ensure that animal numbers are adequate.

We are fully committed to reduction, nonetheless the exact numbers of animals required will vary with the particular project needs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have many years of experience of parasite culturing and historical yield data from host animals to ensure the minimum number of animals are being used. Due to our experience we have highly optimised the process of culturing and storage of parasites to maintain higher parasite yields. We also utilise the NC3Rs Experimental Design Assistant, VICH and WAAVP guidelines as well as consulting guidelines set by regulatory bodies and any relevant literature to ensure that the minimum number of animals is used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We only produce as many parasite infected animals as we need to maintain our own parasite stock collections or produce as much as is required for a customers' order. We will not unnecessarily use animals in this project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use animal models designed not to produce any clinical signs as far as possible, thus the severity category is within the mild or moderate bracket. We try to use in feed methods of helminth parasite administration to reduce animal stress and we allow a period of time for the animals to become accustomed to procedures before starting, for example, wearing a dung collection nappy prior to the collection starting. Refinement of animal experimentation through defined animal welfare codes covering husbandry conditions, regular health checks, appropriate therapy, defined end points and other areas will be developed. Where necessary we slaughter animals using schedule 1 of the establishment licence (XFD0F8970) or other permitted methods contained within the establishment licence.

The severity levels achieved are predictable and are designed to minimise adverse reactions. We refine and/or validate existing/new animal models/techniques in support of 3Rs initiatives and the purpose of this project.

With ectoparasite infestations, where necessary parasite populations can be partly removed to reduce severity.

Why can't you use animals that are less sentient?

We have to use these hosts due to the nature of our parasites being passaged through their natural hosts. However the design of our experimental models mean that the severity to the animals is maintained at sub-threshold/ mild with helminth infections, and as low as possible for ectoparasite infections.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We minimise suffering by examining each planned culturing activity to identify opportunities to reduce handling by, for example, using experienced and knowledgeable staff, habituating animals to procedures where necessary, meeting or exceeding codes of practice and current guidance or literature suggestions for enrichment.

The animals used are the target species for the desired parasite to be cultured. In addition, animals will have a heightened level of observations immediately after and in the days following administration of any substance and/or parasite. Our standard level of monitoring is a minimum of twice daily. Frequency of observations are increased on an as needed basis based on animal observations and behaviour. For example, observation frequency would increase to hourly, overnight monitoring following the appearance of clinical signs associated with parasitic infection. Observations would normally be general health observations but these are supplemented with clinical observations by our NVS (or similar), monitoring of temperature and other parameters as necessary.

If it is believed an animal is approaching and likely to breach the predetermined severity category then we have a well-established process. This includes seeking veterinary advice from our NVS or another veterinary surgeon, discussions with the NACWOs, PELh, PPLh, PILh and Study Investigator. This group of people decide the best next steps in terms of animal welfare. This could be either, immediate alleviating of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, and increased monitoring if the animal is unlikely to breach the severity limit by the conclusion of the study if appropriate.
If an animal breaches the predetermined severity category for the study then immediate action is taken. This often includes rapid discussions with the NVS, PELh, PPLh and PILh alongside the study investigator. We would also, if possible, contact an ASRU inspector. The course of action could be immediate alleviating of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, maintaining and closely monitoring the animal if the scientific need is justified and the end of the study is within a short time frame, if agreed by an ASRU inspector. All decisions are taken on a study by study basis and will differ with treatments and species.

We will also continually review our procedures and refine as much as possible with guidance from, for example, VICH, EMA, WAAVP, and other relevant organisations and published literature.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for best practice. These guidelines are now seen by most regulatory authorities as the standard by which parasite-related protocols are written, and consider numbers of parasites required for study purposes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly reference the NC3Rs website, published literature, attend relevant conferences and employees are members of The Royal Society of Biology and other relevant organisations.

112. Evaluation of new treatments for polycystic kidney disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Kidney, Treatments, Cysts, Genetics

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's the aim of this project?

The overall aim of the project is to identify novel therapeutic strategies to treat Polycystic kidney disease. Treatments are tested for their ability to reverse or slow down kidney cyst formation and improve overall kidney function. The aim is to also minimise adverse effects compared with current therapies, in order to improve the quality of life for PKD 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?

Polycystic kidney disease (PKD) is a debilitating disorder characterised by progressive kidney cyst formation leading to end stage renal failure. It affects about 70000 adults and children in the UK and accounts for 1 in 8 people in need of a kidney transplant. This work is expected to provide new information on mechanisms regulating kidney cyst formation, which will be used to identify new therapies with better efficacy and fewer side effects. As well as improving symptoms of the disease, the hope is to delay the progression to end stage renal failure, therefore negating the need for a kidney transplant.
What outputs do you think you will see at the end of this project?

This work is expected to provide new information on mechanisms regulating kidney cyst formation, which will be used to identify new therapies with better efficacy and fewer side effects. This project is key to triaging compounds discovered within the company for efficacy and tolerability in the whole animal system, a key step before potential testing in man.

Who or what will benefit from these outputs, and how?

Ultimately kidney disease patients will benefit from this project, improving their quality of life. This will hopefully translate as improving symptoms of the disease and delaying the progression to end stage renal failure, therefore negating the need for a kidney transplant. Data generated will also feed back into the research cycle to optimise drug candidates going forward.

How will you look to maximise the outputs of this work?

Findings will be made available to other company scientists at presentations and internal meetings to further our understanding of the disease. We will take as many tissues as possible at study end which will be made available to other company researchers, allowing the maximum amount of data to be generated from the animals.

Species and numbers of animals expected to be used

- Mice: 22000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 both neonatal and adult mice. It is known that cysts are already present in the foetus in severe cases of PKD, so it is therefore important to study how age affects disease progression, and how intervention with a therapeutic can affect the symptoms at these different life stages.

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.

Typically, what will be done to an animal used in your project?

Mice will typically receive injections of therapeutic to investigate the effects on reducing cyst burden in the kidney. These injections will be typically sub-cutaneous as we need to dose over the course of the disease. This may be for a couple of weeks in a short aggressive model, or up to 3 months in a longer chronic model. Total kidney volume can be measured non-invasively and longitudinally every month, to track the disease course.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice are expected to experience transient pain from injection of therapeutic agents. Imaging techniques to track disease progression are painless, but animals are briefly anaesthetised for these procedures so imaging is performed as infrequently as possible. As kidney disease develops and affects the wellbeing of the animal, especially in the quicker models, weight loss may become a concern. This is monitored and the animal humanely culled if it drops below a threshold.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice - Moderate - 40% Mild - 60%

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?

Polycystic kidney disease (PKD) animal models are required to assess the effect of a test compound on kidney cyst formation, total kidney volume and global renal function.

Cell and ex-vivo assays can give a good indication of the potential ability of a compound to modulate the formation of cysts, but they cannot fully predict in vivo efficacy on global renal function or disease progression. In vivo models are therefore an absolute necessity to relate in vitro data to efficacy in order to predict a potential clinical benefit. In addition, how the drug will behave in the body and how it will be broken down, driven by absorption, distribution, metabolism and elimination, cannot be accurately modelled in vitro.

Finally, for PKD, proven in vivo efficacy data is a prerequisite of the regulatory bodies who have the authority to approve or reject a new drug application.

Which non-animal alternatives did you consider for use in this project?

Alternative approaches within the company, including the use of kidney organoids and cell culture to screen compounds for efficacy and toxicity. Human diseased kidney tissue has also provided useful project information. Ex-vivo embryonic kidneys have provided useful screening information but require an animal initially to generate them.

Why were they not suitable?

Cell and ex-vivo assays can give a good indication of the potential ability of a compound to modulate the formation of cysts, but they cannot fully predict in vivo efficacy on global renal function or disease progression. In vivo models are therefore an absolute necessity to

relate in vitro data to efficacy in order to predict a potential clinical benefit. In addition, how the drug will behave in the body and how it will be broken down, driven by absorption, distribution, metabolism and elimination, cannot be accurately modelled solely 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?

This estimate is based on previous in-house studies. We have extensive experience in using these models and can therefore accurately estimate the number of animals we will need per group to obtain statistically valid results. As we generate more results, we will continually assess group sizes 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 keep sample sizes to a minimum.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have made use of all local support including our statistician and the wealth of knowledge from my in vivo colleagues who have years of planning experience. Ultrasound monitoring and measuring of disease burden longitudinally in the same animal has also reduced animal usage, as well as increasing statistical power.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our use of cell culture systems will reduce the number of experiments that need to use live animals. All compounds will be triaged as much as possible through in vitro screens so only the ones with the most favourable chance of success are progressed to the in vivo models. We will conduct pilot experiments where necessary to ensure that our experimental systems are optimised before conducting full cohort studies. Colleagues with expertise in drug metabolism work closely with us, so we can share tissues with them to reduce the total number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Various polycystic kidney disease models will use mice from neonates up to adults, with varying levels of disease severity (from acute to chronic). In the acute model the effect of

treatment on kidney volume and cystic burden is measured from the tissues at a set disease endpoint, allowing for a rapid assessment of therapeutic benefit without long term suffering. Longer chronic models better recapitulate the disease progression in humans, and are therefore necessary to ascertain that our approaches will lead to superior patient benefit than the standard of care in the clinic, tolvaptan. To minimise harm and animal numbers per study, disease progression is monitored longitudinally with ultrasound. Models use human cell implants to assess the efficacy of our therapeutic approaches on human cells, in order to improve translation to man. Before implanting cells under the kidney capsule, which is more invasive, a subcutaneous approach will be used.

Why can’t you use animals that are less sentient?

The models used are based on the gene mutations that have been shown to cause PKD in human patients, therefore improving the likelihood of translation from efficacy observed in these models to the clinic. Moreover, studies in rodents deliver robust, reproducible data and so it is often unnecessary to evaluate efficacy of new compounds in higher species. As we need to monitor disease progression chronically over time it isn't appropriate to perform studies in terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At all stages of the project, we will conduct welfare assessments on the animals to evaluate the impact of procedures. This will determine if any aversive procedures can be minimised without adversely affecting scientific outcomes. We will also seek advice from the named veterinary surgeon and named animal care and welfare officer, as well as other scientists working in the in vivo area. Specific health monitoring paradigms are in place for all models performed under this licence.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will refer to published guidelines issued by NC3Rs and liaise with in vivo colleagues, our named veterinary surgeon and named animal care and welfare officer, to ensure our studies are performed in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep up to date by reading scientific articles/attending conferences and liaising with colleagues. I will also liaise frequently with the named veterinary surgeon and named animal care and welfare officer who will give advice on how to implement any advances.

113. The effect of dietary protein restriction on the development of the neuromuscular system

Project duration

2 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

protein restriction, skeletal muscle, ageing

Animal types	Life stages
Mice	aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This study is using an established model of reduced pre-natal (i.e. maternal) and postnatal protein intake in mice in order to determine the effect of reduced in utero or early post-natal protein intake on neuromuscular junction structure, muscle fibre number, mass and function in the offspring during ageing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In utero and early postnatal protein restriction are associated with poor ageing, reduced muscle mass and function (termed sarcopenia when it reaches clinically relevant severity) and a shortened lifespan but little is known about the mechanisms involved. Our hypothesis is that in utero and early postnatal protein restriction adversely affects muscle

homeostasis leading to a reduction in the myofibre number and size; this in turn leads to premature sarcopenia.

The research proposed is of high importance as the outcomes of this study will lead to a greater understanding of the role that diet plays on the processes underlying the loss of muscle mass and neuromuscular function in older individuals and hence to the logical development of interventions to correct these processes.

What outputs do you think you will see at the end of this project?

The findings of this project will be published in peer reviewed journals and presented by the applicant at national and international scientific conferences targeted to research audiences interested in nutrition, ageing and frailty in addition to those studying other disorders associated with denervation and muscle atrophy such as motor neurone disease (ALS), diabetes, incontinence.

Who or what will benefit from these outputs, and how?

This research will contribute to the body of scientific knowledge on the processes underlying the involvement of malnutrition in age-related weakness and loss of function of skeletal muscle fibres and motor units and will be of particular interest to those scientists and clinicians researching nutrition, frailty and weakness in the elderly. The current research is also likely to be of interest to researchers studying epigenetic changes related to ageing. In the longer term and in combination with other studies it may lead to a redirection of the approach taken in interventions to maintain skeletal muscle function in elderly humans and animals. It will therefore be of interest to nutritionists, neuromuscular physiologists, gerontologists and clinicians interested in maintaining health in the elderly.

How will you look to maximise the outputs of this work?

The applicant is committed to build and sustain collaborations and currently has multiple active national and international research collaborations. The applicant has published high impact manuscripts in this area and is frequently invited to present at international conferences on skeletal muscle and ageing (e.g. European Society for Free Radical Research, British Society for Research on Ageing). The applicant also regularly gives lay presentations to the public and schools in association with charities such as Age UK and provide material for relevant local and national TV and radio programmes.

Species and numbers of animals expected to be used

- Mice: 12

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These animals continue from a previous study. If not used, all mice will be wasted.

We will study the effects of in utero and post-natal protein restriction on neuromuscular structure and function in older mice.

The mouse is the lowest species that demonstrates comparable nerve-muscle interactions. Hence we have rejected any other animals models that might reflect dietary restriction effects approximating to the same developmental stage in humans.

Most of the intrinsic and extrinsic changes regulating muscle ageing in humans have also been observed in rodents, indicating that mice are models of human sarcopenia. Ageing humans and mice show loss of muscle fibres with ageing, particularly in the muscles proposed for use in this study, including the gastrocnemius, tibialis anterior (TA) and extensor digitorum longus (EDL). Denervation also contributes to loss of muscle mass in humans and rodents and loss of muscle fibres is associated with a loss of motor units in humans and mice.

Typically, what will be done to an animal used in your project?

These animals continue from a previous study and are currently ageing. Mice will be culled at 18 and/or 24 months of age. Some mice will undergo grip strength measurements. This will allow us to determine whether mice exposed to a low protein diet in utero or during lactation develop early onset of sarcopenia.

What are the expected impacts and/or adverse effects for the animals during your project?

Some mice on the low protein diet may show signs of ill health or reduced weight loss in comparison to their respective controls.

Ageing phenotype: Hair loss, sometimes due to over grooming is common in ageing mice especially if they are a BL6 strain or background. It is also noted that genetically-modified transgenic mice with high levels of fluorescence can have problems with skin conditions. This is often recognised by red, inflamed skin that may break due to scratching. Treatments include cleaning area with 1% chlorhexidine solution and applying green clay to soothe skin or prescribed drugs (for example: anti inflammatory, steroid based creams) as prescribed by the vet.

Loss of general condition due to ageing process includes loss of muscle tone, tumours and development of cataracts. Developments of any tumours are a humane end point and the animal must be culled. Cataracts are fine as long as the animal can still eat and drink freely. Such welfare issues can be recognised by piloerection, staring coat, weight loss, slightly hunched appearance and altered gait but still mobile and able to get to food and water freely.

Other adverse effects may be associated with muscle weakness and resulting (sporadically) in change in movement. These will be monitored for through observing the mice daily, weighing the mice. Any significant changes or moderate changes lasting more than 3 days will result in animal cull by an authorised method and tissue collection. At the end of the protocols, animals will be killed using an authorised method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity is expected for more than 50% 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?

Due to the nature and complexity of the experimental design and in particular the need to reduce protein intake during pregnancy and during lactation of the offspring it is not possible to use human tissue, therefore the proposal requires the use of in vivo studies in mice. In addition, because of the need to study the interplay between nerve and muscle cells and ageing of post-mitotic tissues, it is not feasible to use a cell culture based approach. Nerve-muscle co-cultures remain immature. It is not possible to imitate the innervation of muscle ex vivo. The rodent is the lowest species that demonstrates comparable nerve-muscle interactions. Mice are the lowest vertebrate group possible in this study and the availability of genetically altered mice will provide definitive data necessary to achieve the objectives for this study. Other species have been considered but deemed unsuitable for these experiments, such as Zebrafish, drosophila or nematode.

Which non-animal alternatives did you consider for use in this project?

Cell culture.

Why were they not suitable?

Ageing is a whole organism phenomenon. The ability to examine the effect of age on muscle structure and function is not possible in cell culture. The alternative is the use of primary culture to generate muscle from aged mice. However, this raises the problem of the lack of a suitable model to perform muscle force measurements in culture. In addition, age-related functional decline is due to loss of individual muscle fibres as well as an atrophy and weakness of remaining fibres. The mechanisms responsible for this are not understood and a cell culture approach would not be possible 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?

These animals are currently ageing in order to complete a previous experiment that begun on a previous project licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of mice were minimised by performing multiple assessments/analyses on the same mouse. The original proposal was designed with the help of statistical advice to minimise the number of mice used overall.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

These animals will complete previous experiments that begun on a previous project licence where appropriate power calculations and statistical advice was sought. Tissues from these mice will be made available to other researchers and data will be shared.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are chosen for these experiments due to the similar physiology between mouse and human muscle. The mouse is relatively short-lived with an average lifespan of 2 - 3 years, therefore the effect of age on muscle structure and function can be fully documented over a relatively short timescale.

Studies will be undertaken in wild type (WT) mice or mice expressing fluorescent proteins in neuronal cells (with no expression in non-neuronal cells and no apparent toxic effects). The transgenic mice (purchased originally from commercial breeders) express yellow or cyan fluorescent proteins in high levels in motor and sensory neurons, as well as in subsets of central neurons. These lines provide a strong and specific marker of axons and expression is strong from mid-gestational stages, showing 100% expression in motor neurons that allows detailed visualisation of skeletal muscle innervation using confocal microscopy. We have successfully used such transgenic mice in the past.

Why can’t you use animals that are less sentient?
This project will use mice during ageing to complete work from a previous study. Mice are the lowest vertebrate group possible in this study and the availability of genetically altered mice will provide definitive data necessary to achieve the objectives for this study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

A number of check points for minimising animal suffering are included in the protocol design, including clear end points and shortest time necessary. The applicant has considerable experience in all the techniques used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The general experimental designs and methods of analysis of the results have been discussed with University's statistician. To ensure best practice and if unexpected adverse effects arise, advice from the "Home Office Animals in Science Regulatory Unit" will be sought.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant regularly attends workshops organised by NC3Rs in order to stay informed.

114. Sprayed slow-release analgesic for application during abdominal surgery

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Vesicles, Hydrogel, Analgesic, Peritoneum, Abrasion

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 investigate the safety and efficacy of a single-dose painkiller (analgesic) formulation for its use during abdominal surgery, avoiding significant side effects of commonly used analgesics.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Surgery on the abdomen is painful. It is usually treated by various painkillers (analgesics), which can cause secondary problems (drowsiness, constipation, poor mobility), leading to a slow recovery. Local painkillers can provide short-term pain control at wound sites but do

not target the area of surgery within the abdomen. There is a need for better strategies for controlling pain within the abdominal cavity. Therefore, we will develop a long-acting pain relief spray that remains active at the surgical site in the abdomen. Such a spray should be administered as a single dose directly onto the surgical site and provide continuous pain relief for 5 days.

What outputs do you think you will see at the end of this project?

Our spray analgesic will provide constant pain relief during the whole recovery period following abdominal surgery. This technology can be adapted to any open surgical procedure or site of trauma and adapted for the slow release of other drugs, i.e., chemotherapy. Outputs from the research will add to the general knowledge about alternative methods for providing safe and effective pain relief medication; this information will be published in academic journals to improve scientific knowledge.

Who or what will benefit from these outputs, and how?

The primary beneficiaries from the outputs will be patients undergoing laparoscopic surgery for benign and malignant abdominal conditions. Our sprayed formulation will provide constant pain relief during their hospital stay leading to quicker recovery, fewer complications, and faster return to normal function. Secondary beneficiaries will be healthcare providers. It is estimated that our pain relief medication will help reduce the length of time that patients spend in the hospital from 5 to 3 days, resulting in cost savings of ~£1,300/patient or ~£1.7billion/year to the NHS.

How will you look to maximise the outputs of this work?

We will disseminate the findings to relevant forums through presentations to surgical and pain associations in the UK and publish the results in peer-reviewed journals. The group will undertake various outreach activities to ensure that the general public is aware of the research. This will include presentations to “Pint of Science” and “Be Curious” events organised through the local research institution. To ensure lay people easily understand the relevance of the work, we will use modern forms of communication, such as Visual Abstracts. Engagement with public and patients will be central to the design and conduct of the research to ensure that it remains directly relevant to end-users.

Species and numbers of animals expected to be used

- Mice: Optimisation experiments will be performed on 40 C57BL/6 mice to determine the sample size for the definitive study. The final number of animals for the definitive study is unlikely to exceed 200 in total.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice are a good choice for animal experiments because they have many similarities to humans in terms of anatomy and physiological responses. Less sentient animals cannot

be used because they would not help us to assess the pain relief capabilities of the formulation.

Typically, what will be done to an animal used in your project?

The animals will go through a simulation of abdominal surgery. Animals will undergo laparotomy (opening of the abdomen) under general anaesthesia and a 1cm2 abrasion made to the peritoneum to simulate surgical trauma. Half the animals will have the analgesic formulation applied to the traumatised area; in the other half the area will be left untreated. The abdominal wound will be closed and the animal allowed to recover. Animals will be monitored to ensure their safe recovery and to assess their pain sensations with a validated scoring system. All animals will be euthanized at the end of the 5 day observation period to obtain tissues and fluid samples for further analysis.

A small number of animals in the optimisation experiments will undergo application of gel carrier alone (without painkiller) to confirm that application of the gel alone does not influence postoperative pain. This would be most unlikely given that the gel is a biologically inert material. Inclusion of a gel carrier arm has not been added to the main study as it is unlikely to add any meaningful information and would only serve to unnecessarily increase the sample size of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Possible adverse effects include the failure to provide pain reduction, a bacterial surgical site infection, wound infection and dehiscence, and organ dysfunction resulting from surgery. We will closely monitor for adverse effects and trained personnel will take immediate action to avoid unnecessary harm to the animals. A validated postoperative assessment scoring system will be used to grade the severity of complications and determine a humane end-point for euthanasia (described in the stop/go section). All the animals will be humanely sacrificed at day 5 to obtain different samples from fluids and organs for further analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The proposed methodology is of moderate severity because it involves abdominal surgery. We will require 40 mice for initial optimisation experiments to determine the sample size for a definitive experiment. It is not expected that the final number of animals for the definitive study will exceed 200 mice.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?
It is only possible to test the safety and efficacy of our formulation in a living biological model which experiences pain. We have previously assessed some inflammation (pain) markers associated with wound healing in the laboratory using cells. These experiments have shown promising results without any bystander toxicity. We need to verify that the results obtained are reproduced in a biological model.

Which non-animal alternatives did you consider for use in this project?

We have previously undertaken in vitro optimisation studies using human cells to detect different inflammation (pain) markers. There is nothing further we can do in vitro to advance our technology. We have considered the option of using less sentient animals, but these will not allow us to properly assess our primary end-point – pain reduction.

Why were they not suitable?

Human cell lines can only provide surrogate markers of pain control, i.e., reduced inflammatory mediators. While this can be considered a good indicator of pain reduction, it cannot be fully verified because pain is a complex sensation in biological systems. An animal model is now needed to understand the safety and efficacy of the formulation.

Reduction

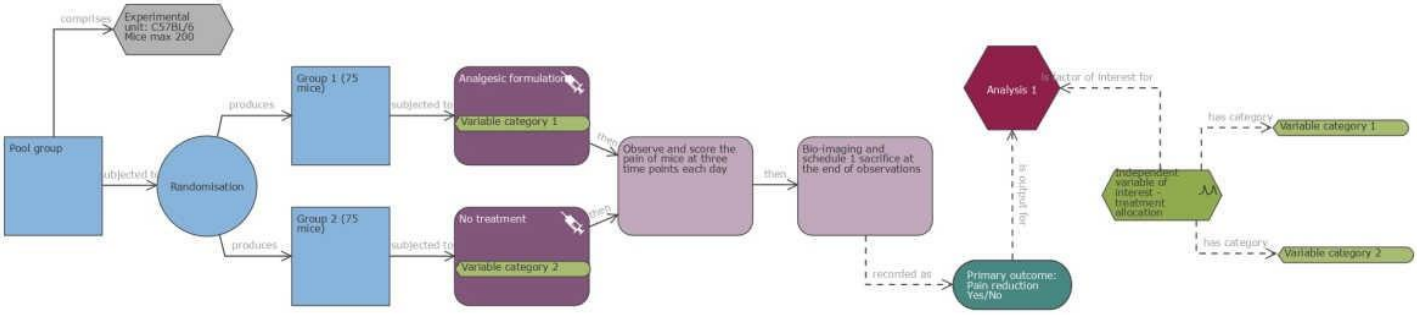
Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may 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 discussed the design of our experiments with biostatisticians to obtain reliable and reproducible data with the fewest possible number of animals. We will first perform optimisation experiments in 40 animals, which will inform the sample size for a definitive study. Based on a 30% reduction in Grimace score on postoperative day 1 or 2 in favour of the intervention group we anticipate a total sample size of approx. 150 animals (75 intervention; 75 control) to demonstrate a statistical difference at 80 power and 5% significance level.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We utilised the NC3Rs experimental design assistant to guide the numbers needed to demonstrate the safety and efficacy of the novel sprayed analgesic formulation in an abrasion mouse model. We have adhered to the 3Rs principles, and up-to-date ARRIVE guidelines to design the experiment. The ideal study design to evaluate efficacy is a randomised controlled trial. In line with the 3Rs of animal research, there will be two groups, the analgesic formulation alone and no treatment, to keep the numbers of mice to a minimum.



What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Training for the model will be performed on approximately 6 schedule 1 mice. Optimisation studies using 40 mice (20 intervention group; 20 control group) will establish the required number of animals for the definitive study. This will include a small number of animals to confirm that the gel carrier does not influence postoperative pain scores. The ideal study design to evaluate efficacy is a randomised controlled trial. In line with the 3Rs of animal research, there will be two main groups, the analgesic formulation and no treatment, to keep the number of mice to a minimum. Randomisation & Blinding: Mice will be randomised using block randomisation through SealedEnvelopeTM at a 1:1 allocation ratio. All mice and samples will be labelled such that researchers assessing the effects of the treatment and analysing the results will be blinded.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been chosen for the experiment because they are ideal animal models for biomedical research and comparative medicine. They have many similarities to humans in terms of anatomy and physiology. We will use an abrasion model for the experiments adapted from Brodsky et al. and Kraemer et al. This model allows us to simulate the trauma of abdominal surgery without causing harm to vital organs. It is believed this model provides the least pain, suffering, distress, and lasting damage to the fewest number of animals, whilst still enabling us to adequately assess pain reduction associated with our formulation.

Brodsky, J. A., Brody, F. J., Endlich, B., Armstrong, D. A., Ponsky, J. L., & Hamilton, I. A. (2002). MCP-1 is highly expressed in peritoneum following midline laparotomy with peritoneal abrasion in a murine model. Surgical Endoscopy, 16(7), 1079–1082. doi:10.1007/s00464-001-8335-z

Kraemer, B., Wallwiener, C., Rajab, T. K., Brochhausen, C., Wallwiener, M., & Rothmund, R. (2014). Standardised Models for Inducing Experimental Peritoneal Adhesions in Female Rats. BioMed Research International, 2014, 1–8. doi:10.1155/2014/435056

Why can’t you use animals that are less sentient?

Less sentient animals would not help us assess the pain relief capabilities of our analgesic formulation due to their lack of nervous system. If the mice were terminally anaesthetised, we would not be able to measure the healing effects of the intervention at the required post-operative stage.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be reviewed regularly by the research team and staff from the Animal Facility to ensure animals do not experience excessive pain. A validated pain scoring system will be used - the Grimace Score. Oral analgesia (buprenorphine) will be given to animals, in both the intervention and control groups, with a Grimace Score of >/= to 3 out of 10 and the total daily analgesic requirement recorded. General animal well-being will be monitored using the validated Abdominal Surgery Post-operative Severity Assessment. At the first sign of a point of no return (>20 points on the scoring system), the animals will undergo schedule 1 killing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs & ARRIVE Guidelines and guidelines local to the research institution will be followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have reviewed the NC3Rs website regularly to ensure that our practice is up to date, for example, the recent change to the ARRIVE guideline recommendations (du Sert et al., 2020). We are also on the local animal housing mailing lists that distribute urgent updates that are reviewed regularly.

du Sert, N. P., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., … Würbel, H. (2020). Reporting animal research: Explanation and elaboration for the arrive guidelines 2.0. PLoS Biology (Vol. 18).

115. Novel brucellosis vaccine development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Brucellosis, Vaccine

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To evaluate the potential efficacy of novel advantageous vaccine candidates that could protect livestock against brucellosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Brucellosis remains one of the world's most significant diseases of livestock, incurring a huge burden on both animal and farmer's welfare and a significant financial cost to the industry. Two of the three main causative bacterial species, Brucella abortus and B. melitensis are in the top 5 causes for livestock units lost for cattle, buffalo, camels, sheep and goats. Additionally, brucellosis is a significant disease in swine, largely caused by B. suis. Although there are three brucellosis vaccines in existence, they have considerable shortcomings. Despite these being available for decades, brucellosis is far from retreating and is a re-emerging disease across large swathes of the world. It is also one of the world's most significant zoonoses with an estimated annual incidence of between 5 and

12.5 million human cases. There is no human vaccine or internationally recognised vaccine for swine.

There are many problems with the current vaccines for cattle (B. abortus S19 and B. abortus RB51) and sheep & goats (B. melitensis 16M). These are live attenuated vaccines, but all possess residual virulence. Vaccination of pregnant animals frequently causes abortions, and this is a major cause of farmers choosing not to vaccinate. The vaccine strains are also pathogenic to humans. In addition to safety issues, there are additional costs as they must be cultured in specialist high containment laboratories. Also, during administration needlestick injuries frequently result in infection, which is compounded by the live vaccine strain's resistance to frontline antibiotics used to treat Brucella infection in humans. Two of the vaccines (B. melitensis 16M and B. abortus S19) generate positive results in the diagnostic tests used for brucellosis disease surveillance, making it very difficult to differentiate between infected and vaccinated animals. This leads to the slaughter of many healthy animals, which in turn affects uptake by farmers. This situation is a major impediment to disease control as the current vaccination regimens alone do not create sufficient pressure for disease eradication, which now relies on test & slaughter. Furthermore, the governing international organisation (the OIE) will not certify a country as officially free from brucellosis if vaccines have been used within the past three years. Under these conditions it is very difficult for brucellosis to be eradicated and one reason why it persists so abundantly in many areas of the world. Additionally, the B. melitensis 16M vaccine is unstable and will spontaneously change into a form that loses the major immunogenic component, so becoming less protective. The B. abortus RB51 vaccine is a mutant strain which has also lost this component and as a result is less effective. The current vaccine for sheep and goats is sufficiently problematic that AgResults (funded by the Gates Foundation) have funded a $30 million 'Brucellosis Vaccine Challenge Project' to improve upon it.

The basis for this project is the development of a brucellosis vaccine that is safe, protective, lacks cross-reactivity with diagnostic tests and has the commercial potential to lead to the production of an improved vaccine that can resolve many of the issues described above and provide an attractive option to facilitate the eradication of this disease.

What outputs do you think you will see at the end of this project?

Information on whether the vaccine candidates are immunogenic and protective in a mouse model will be the primary output.

This information will likely provide at least one scientific publication.

Who or what will benefit from these outputs, and how?

If the results for a vaccine are promising, it will be taken forward by our commercial partner for further testing in livestock within 24 months, which could give rise to the production of a commercially viable licenced vaccine within 10 years. This would provide benefits to the target livestock industry globally, such as improved animal welfare, increased production and improved wellbeing for farmers. With its potential to reduce the prevalence of brucellosis in livestock, this would also help reduce its huge annual incidence in humans. As this novel brucellosis vaccine concept has not been previously assessed, regardless of the outcome, the data produced will help inform ourselves and the scientific community of its application. This would help in the further rational development of brucellosis vaccines.

How will you look to maximise the outputs of this work?

Through our partnership with a global veterinary pharmaceutical company, with whom we have also been developing the vaccine production methodology to help ensure commercial viability, the outcome of this work has every chance of leading to a licensed vaccine. We aim to publish both successful and unsuccessful results in open-access peer reviewed journals. Results may provide information to allow us to modify the vaccine to improve the scientific outcome of follow up work.

Species and numbers of animals expected to be used

- Mice: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been selected here as a first line screen for vaccine immunogenicity and protective efficacy. Other animal models (such as rats, rabbits and guinea pigs) were evaluated, but mice remain the most relevant and standard small animal model for brucellosis research. A large body of scientific publications use the mouse models, which allow the data generated in this study to be more easily translated into potential observations in the target species (cows, sheep, and goats) for which this vaccine is being developed. This track record also enables virulent challenge studies to be conducted with knowledge of the outcomes and influencing factors to ensure severity is minimised to obtain the required data output, which is very reproducible in this model. As reviewed by Grillo et al. (2012), mice do not show clinical signs of suffering on challenge with standard doses of Brucella, which means that they are able to control the replication and progressively reduce the bacterial burden. Experimental challenge in the target species would lead to an increase in the potential for pain, suffering or distress because the model requires the challenge to be conducted during pregnancy using abortion (in the third trimester) as a readout. Therefore, initial screening in mice is the preferred option for new vaccines before committing to the experiments required in these target species.

Adult mice (>8 weeks old) will be used to ensure their immune system is mature. These will be sourced as Specific-Pathogen-Free (SPF) to reduce data variability due to other infections. Only females will be used to further reduce variability and allow group housing. The in-bred strain, BALB/c, have been selected as the most frequently used for brucellosis vaccine testing and have been recommended for this purpose (Grillo et al. 2012).

Typically, what will be done to an animal used in your project?

Animals will be injected (subcutaneously) with a vaccine, typically involving three separate immunisations 2-4 weeks apart. Blood samples will be taken (not exceeding 15% Total Blood Volume (TBV) within 28 days and up to 10% TBV per sampling) from superficial vessels prior to each immunisation for immunological assessment.

Subsequently animals will be euthanised, a blood sample taken by aortic incision or cardiac puncture during terminal anaesthesia and the spleen isolated for additional

assessment of immune responses (total duration ~50 days). Alternatively, four weeks after the last vaccination, animals will be challenged by injection with virulent Brucella spp. bacteria and euthanised to assess bacterial burden in the spleen, typically 2 weeks later (total duration ~80 days).

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects during the immunisation period may include mild transient discomfort or distress from the injections and blood sampling. The administered vaccine formulation may consequently induce an inflammatory response and localised swelling causing general discomfort, which may last a few days (equating to no more than moderate severity).

Apart from the mild transient discomfort of the injection, challenge with virulent Brucella spp. is not expected to cause adverse effects as the infection is noted to not cause any clinical signs in mice when using doses no greater than the large effective range of between 104 – 5x105 Colony Forming Units (Grillo et al. 2012).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild (25%)
Moderate (75%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no alternatives to testing of brucellosis vaccines in vivo. There are presently no in vitro assays that can confidently predict vaccine efficacy and be accepted as strong evidence to allow investment in testing in the target livestock.

Which non-animal alternatives did you consider for use in this project?

Ex vivo primary cells Invertebrates
biomimetic tissue culture organoids

Why were they not suitable?

Ex vivo primary cells from animals cannot effectively mimic the generation of an immune response to immunisation or a response to challenge infection due to the complex interplay of many parts to the system in vivo.

Invertebrates lack an acquired immune system, which plays a key role in the protective immune response in the target species. Although insects such as the larvae of the wax moth show potential for studying pathogenesis of some infectious diseases, it is not yet clear that they can be used to effectively evaluate vaccine-induced immunity.

There has been some progress in the development of organoids which mimic individual elements of the immune system, such as the lymph node. Whilst these can potentially provide a surrogate for challenging animals (only after addition of cells/antibodies from vaccinated animals), they have so far been developed to mimic humans as the target population and we do not have a developed and validated equivalent model for our target livestock population.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Currently it is planned that one vaccine immunogenicity study will be conducted to assess one vaccine candidate against an earlier formulation tested in a previous study, probably requiring 5 groups of 10 mice (50 total). If deemed sufficiently immunogenic, a challenge study will be conducted to assess protective efficacy. This will likely involve a pilot experiment (probably one cage of 7 mice, each from a different treatment group) followed by the full study probably requiring 7 groups of 10 mice, due to extra control groups needed (77 total).

It is also a possibility that the immunogenicity study is repeated after modification of the vaccine if initial results did not indicate the presence of the required specific immune parameters (50 total).

Additional animals may be needed for contingency reasons (123).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design will be based on group sizes that should show significant differences so that experiments will not need to be repeated because results were inconclusive. To provide more statistical power by maximising the number of experimental units, the group housed mice will, where possible, be in cages containing animals representative of all the treatment groups. The number of vaccine formulations that were going to be tested has been reviewed and minimised to only what we deem as essential to answer our objective. Although we have never conducted a challenge with Brucella spp. before, a meta-analysis of the published literature has allowed us to be confident that when using the experimental parameters used by others, we can relatively confidently

anticipate the outcome in positive and negative control mice meaning only a small-scale pilot study will be needed. These data have also provided us with a good estimate of the likely variation to be expected so that a power analysis can be used to estimate the correct number of animals more accurately, which could otherwise be over-estimated. Statistical input for studies will be sought to ensure validity of data from the organisations bio-statisticians as well as using the NC3R's experimental design assistant. In-bred mice (BALB/c) will be used to minimise data variability and so reducing the number of animals needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will likely conduct a pilot experiment prior to the challenge to help ensure that the results are not compromised and are of the highest quality when the full study is conducted. This is to ensure that all practical hurdles are ironed out due to the need for stringent high biological containment work practices and to ensure the challenge inoculum is sufficiently virulent, due to potential for attenuation in vitro.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Adult mice will be used. These can be readily habituated to handling by humans to help minimise stress, including during invasive procedures. As a key role in this, we will follow the latest advancement in handling techniques and use non-aversive methods such as tunnelling. Female mice will be used which will allow animals to be group housed, allowing social interaction. The BALB/c strain of mice which will be used are more amenable to handling than others (e.g.C57BL/6 and DBA/2), which again reduces the likelihood for causing distress to the animal.

Mice do not show clinical signs of suffering on challenge with standard doses of virulent Brucella spp., as they are able to control the replication of and progressively reduce the bacterial burden. As the colonisation of the spleen is very reproducible even within a very large inoculum dose range, protective capacity of vaccines can be easily discerned by an accelerated reduction in bacterial burden, which can be observed as early as 2 weeks later.

Experimental challenge in the target species would lead to an increase in the potential for pain, suffering or distress due in part to the required longer periods of time for study outputs and the challenge conducted during pregnancy using abortion events (in the third trimester) to measure protective efficacy.

The subcutaneous injections of the vaccine and blood sampling conducted on the animals should only involve mild transient pain. Injection of the challenge infection will be

undertaken by the intraperitoneal route. Whilst other routes may be preferable from a refinement point of view, it is the recommended and standard method for Brucella challenge of mice. This is due to the ability to ensure 100% of mice are homogenously infected in the spleen, which would otherwise lead to more data variability and potentially the need to repeat experiments. Intravenous injection can also achieve this but is more prone to inoculation errors. Because of its established use, published data on the equivalent use of other routes are scarce and so the inability to conduct an appropriate meta-analysis for a statistical power analysis would also potentially lead to the use of more animals.

Why can't you use animals that are less sentient?

Mice provide well characterised models and immunological tools for such investigations and can provide the required experimental outputs without clinical disease. Species of lower sentience, such as invertebrates, are not yet validated for this work. A competent/mature immune system is required; therefore, fully developed mice are needed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will follow the latest guidance on the strict single-use of needles, in accordance with the recommendations of the NCRs.

After mice receive immunisations they will be monitored more frequently, than the normal twice daily regimen, over the following 48hrs to help spot adverse effects.

Blood volumes will be minimised to only that required to carry out the laboratory tests (and in accordance with the NCRs guidance of not exceeding 15% Total Blood Volume (TBV) within 28 days and up to 10% TBV per sampling). This will allow small single-use lancets to be used to yield controlled volumes of blood which will be collected in proprietary capillary tubes of set volume. This use of 'micro-sampling' coupled with considerate handling, should minimise any harms caused.

To ensure unexpected adverse effects of the challenge infection are noticed as early as possible, a daily clinical scoring system will be used so that animals can be euthanised at an appropriate objective clinical endpoint. The use of a pilot challenge experiment will also minimise any unexpected harms.

All animals are group housed and provided with as much environmental enrichment (toys, shredding material, seeds etc.) as is possible under appropriate biological containment. Environmental enrichment is constantly re-assessed, in discussion with animal technicians, NACWOs and the NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance is obtained from NC3Rs, ARRIVE, IAT, LASA and the RSPCA. 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 stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will have regular contact with the NIO, NACWO and NVS through various forums. This includes during AWERB, pre-study and study wash-up meetings where suggestions for improvements can be implemented. The establishment has a Species Group Care and Use Committee where all PPLhs are invited to attend. Specialist topics are presented and refinements, such as environmental enrichment, are communicated and opportunities for implementation are discussed. By regularly accessing the various online resources accessible via the NC3Rs website, further advances can also be found. Staff often attend external symposia on laboratory animal welfare and feedback from these are reported back. By keeping abreast of current scientific literature on the disease model and the methods for measuring our study outputs, further refinements can be implemented.

116. Breeding genetically altered mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Breeding, Genetically Altered, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

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's the aim of this project?

Overall purpose: Breeding and maintenance of GA mice to support the drug delivery pipelines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 dedicated to the breeding and maintenance of mice whose genome was modified through genome engineering techniques. Therefore, the main direct benefit of this licence is to supply healthy, well characterised (i.e., genotyping, phenotyping), genetically altered (GA) mice to support drug discovery pipelines.

In turn, these mice will contribute to a proven record of accomplishment in bringing novel and effective medicines to the market. Mice generated under this licence will enable us to continue our research efforts to the benefit of patients in the future. In some cases, it is necessary to alter the genome of mice to support drug development. That can be the case when, for instance, i) we need to understand a complex disease or test a complex hypothesis; ii) the drug being developed is made in part of antibodies (large molecules).

Understanding a complex disease:

Complex diseases such as diabetes or cancer are not completely understood. GA mice are instrumental in supporting drug development for such diseases because the modification of their gene helps scientists selectively test the relationship between a drug candidate and a molecule or cell in a model of the disease. Without these mice, it would be very difficult to isolate such molecular relationship from the rest of the microenvironment.

For instance, cells coming from the pancreas of GA mice were used to select some suitable drug candidates for diabetes. Once selected, these drug candidates were administered to GA mice missing specific receptors to confirm the involvement of the receptors and their susceptibility to molecular influencers in diabetes.

Cancer is a complex disease resulting from abnormal cell behaviour during the process of cell renewal. This leads to uncontrolled proliferation of cells, the stimulation of new blood vessels (angiogenesis) and the migration of tumour cells to distant sites within the body (invasion and metastasis). The immune system has the greatest potential for the specific destruction of tumours with no toxicity to normal tissue and for long-term memory that can prevent cancer recurrence. Immuno-oncology research has provided evidence that tumours are recognized by the immune system and that their development can be stopped or controlled long term through a process known as immunosurveillance.

The immune response to tumours is variable, which in some cases may be down to a poor recognition of the tumour' by the immune system. The aim of immuno-oncology drugs is to enhance tumour cell killing by increasing recognition and responses of the immune system to the tumour. Such drugs cannot be designed and developed using traditional mice because the immune system of the mice would interfere with the identification, optimization and testing of relevant large molecules. Using GA mice helps us ensure that the tumours and the environment mimic relevant aspects of the human immune system, assess therapy efficacy, dissect the impact of the tumour microenvironment, and evaluate mechanisms of drug resistance. This is for instance the case of mice belonging to the Pten or ODIn GA strains on this licence.

Generate large molecule therapies:

GA mice have already supported the development of many drugs and drug candidates. Examples of this would include the humanized mouse (ie, a mouse whose genome contains a section of a human gene) for the interleukin 13 (IL-13), which helped with the deeper understanding of some aspects of the skin inflammation. In turn, these mice contributed to supporting the development of monoclonal antibody drugs such as tralokinumab, for eczema.

Another example would be anti-IL 33 monoclonal antibodies, used to treat diseases such as asthma and chronic obstructive pulmonary disease (COPD). These antibodies are currently being trialled in people but beforehand, GA mice helped the generation and understanding of pathological pathways for these diseases.

What outputs do you think you will see at the end of this project?
Short-term: Production of high-quality GA mice or material for research in biomedical sciences.

Mid-term: New knowledge can emerge from the breeding activities: welfare/phenotype information, deeper understanding of fundamental physiological and pathological pathways, understanding of factors influencing the predictivity, replicability and reproducibility of in vivo models.

Long-term: The mice produced on this licence will be used under other PPLs (or equivalent authorities) to support the drug development pipelines. Consequently, new drugs for oncology, respiratory, cardiovascular, metabolic or renal pathologies are expected to be developed as a result from this licence. Similarly, new routes of administration, or refined drugs with less side effects can also be an output from this work in the long term.

Who or what will benefit from these outputs, and how?

Short-term & mid-term: The scientific community will be the main beneficiary because we are committed to sharing and publishing all scientific outputs of interest to the scientific community. Publication might need to be delayed until the IP is delivered/secured.

Mid-term: Publication of scientific outputs coming from the use of these mice across preclinical pipelines.

Long-term: Drug delivery for patients – see previous section.

How will you look to maximise the outputs of this work?

The knowledge gained through constant re-evaluating of our breeding strategies feeds directly into training courses that ran several times a year by the team on managing mouse colonies. This knowledge will also be disseminated across our global in vivo community as part of our commitment to our sustainability strategy.

All new lines have welfare information gathered and the archive is annotated with any relevant information so it can be passed on to future researchers. Any specific welfare issues or phenotype of interest to the community will be published as soon as possible without compromising IP work.

Species and numbers of animals expected to be used

- Mice: 40000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are needed for this project as it is a service licence to supply genetically altered mice to our research programmes. There is no need to age mice on this licence, but we will need mice at all other life stages for breeding.

Mice bred on this licence will be used to supply projects that have been through harm benefit analysis. We expect the majority of the mice produced on this PPL to be used in

the UK for work licenced by ASRU after AWERB scrutiny. More rarely, mice could be sent elsewhere for use, where licencing and Harm Benefit Analysis takes place under local legislation.

Whilst most of our animal research is undertaken at our own facilities, to supplement our internal capabilities and expertise, we outsource a proportion of our animal research to a variety of academic and commercial partners. We contractually require these organisations to comply with high ethical standards and we undertake a range of assurance and due diligence activities to ensure our expectations are being met.

Typically, what will be done to an animal used in your project?

Mice will be born, they will have an ear clip taken for identification and genotyping and then they will be socially housed in a cage with environmental enrichment. Mice of suitable genotype, age and sex will then be transferred to another licence/project, kept as stock for potential future breeding, or bred with other genetically altered or wild type mice. Any mice not moved on to other projects will be killed by a schedule one method or through a procedural route for the collection of tissues.

Sperm, eggs and embryos may also be harvested after killing by schedule one to ensure the backing up of the lines, prevention of genetic drift and minimise the number of animals bred but not used.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of mice on this licence will not experience any adverse effects. Thanks to the knowledge accumulated through the predecessor of this licence and the local expertise, we know that most of the lines bred on this licence are not prone to develop any adverse phenotype. For lines where adverse effects are unknown, new lines, or crossing certain combinations for the first time, mice will be carefully monitored. It is not the intention to keep mice with adverse effects on this licence, since for breeding it is preferable that all mice are healthy. Mice will only be kept if they are needed for breeding; the adverse effects are unavoidable (e.g. part of the phenotype, trait cannot be bred out or improved via veterinary/nursing care) and within the humane endpoints.

In cases where individual lines are associated with known adverse phenotype (not exceeding moderate severity), the breeding of such lines will be these will be and bred in the protocol of appropriate severity.

It is possible that some crosses will result in some genotypes being sub-viable or lethal. In these cases, mice of certain genotypes may not be born in expected ratios, or they may die suddenly soon after birth, mostly before weaning. Rarely, mice may die later in life. For lines where there is a known higher than expected mortality rate the expected rate and details will be justified in the protocol of appropriate severity.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

90% or more of mice on this licence will have a severity of mild or below.

10% or less of mice may have a moderate severity due to the appearance of phenotypes whilst still needed for breeding.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are committed to all aspects of the 3Rs, including the replacement of animal models by animal-free technologies where possible. The remit is this licence is limited to the production of genetically altered mice. These mice will then go and be used experimentally under other project licences (or equivalent authorities). No mice will undergo scientific procedures (other than those strictly related to breeding) under this licence. Therefore, the best way to tackle replacement is to support replacement strategies across the broader pipeline activities. Here are some examples of replacement strategies. Non-animal alternatives are used upstream of in vivo testing during drug discovery. This allows us to perform fewer studies which then have a higher chance of identifying a suitable new drug molecule. These non-animal alternatives include measurements of the likely efficacy of the agent on target cells and in vitro assays to determine potential metabolism and pharmacokinetics of compounds and, where appropriate, toxicity assessments in cell-based assays. More complex assay systems, such as co- culture assays and 3D tumour cell assays are used where the biology is more complex and cannot be replicated by cultures in 2D culture. In oncology, we are also further investigating the potential of tumour slice models to profile compounds where this may help answer specific scientific questions. Only compounds with good potency, efficacy and suitable predicted properties for in vivo testing are selected to be profiled in mouse models.

Cancer, respiratory and metabolic diseases have a complex pathophysiology with multiple components interacting to manifest the disease. Therapeutic agents target specific biochemical responses or physiological mechanisms that in vitro systems cannot replicate. Individual mechanisms can be probed in vitro, and we conduct extensive studies to characterise these as far as possible before conducting in vivo experiments. Activity in particular cell types cannot predict the likely in vivo activity given the complexity of issues such as bioavailability, metabolism and elaborate physiological interactions associated with pathological pathways such as tumour development, the tumour microenvironment and the interactions between the tumour and the immune system.

Whilst co-culture systems can address some of these questions, at present we are not able to fully reflect the complex interplay between cells in the microenvironment of the disease. Animals remain needed because no other system yet reflect the full complexity of in vivo biology. For example, the repertoire of interactions between immune cells, nerve cells, complex hormonal systems and organ- specific cell changes cannot be recreated outside of living animals. As such, there is still a need for using the whole animal, hence the need to breed them on this licence.

We continue to explore new in vitro models in our pursuit to reduce and replace the use of preclinical animal models. One rapidly evolving area is the field of microphysiological systems (MPS), which enable human-derived cells to be cultured on bioengineered chips, thereby more closely replicating tissue microenvironments, circulatory flow and cell–cell interactions. It is hoped that MPS will ultimately reduce or replace some safety experiments in animals in addition to enhancing translation, thereby replacing existing in vitro models.

Furthermore, regulatory authorities such as the FDA and EMA require compelling data packages to support the development of new medicines in humans. In vitro potency data are seldom sufficient to provide confidence of efficacy in man, and demonstration of activity (and mechanism) in animal models remains pivotal.

Which non-animal alternatives did you consider for use in this project?

The purpose of this licence is limited to providing a service supplying high quality, characterised mice for controlled and reproducible preclinical studies, where in vivo models remain required, and no animal-free alternative exists. There is no non-animal alternative to breeding. Non-animal alternatives, when they exist, are relevant to subsequent licences, not the present one. No mice will undergo scientific procedures (other than those strictly related to breeding) under this licence. However, here are examples of non-animal alternatives already in use, aiming at reducing the need for genetically altered mice.

Every project goes through intense scrutiny before the decision of using genetically altered animals are used. On this service licence, the reasoning underpinning the decision to breed GA mice will depend on the strain and its expected use.

Example of non-animal alternative for use instead of immunisation of GA mice for antibody generation.

Prior to deciding to conduct an in vivo campaign, we perform due diligence on all targets proposed for antibody generation. If there is precedence for success using in vitro antibody discovery technologies for the target or protein family, then we will not use the in vivo platform. There needs to be a strong rationale for generating in vivo derived antibodies over in vitro, and where we believe in vitro will work will then we always use that technology first and fall back to in vivo if it does not succeed, never parallel track them.

There are particular target types where in vitro technologies are poorly suited to antibody generation, e.g. complex membrane proteins such as G-protein coupled receptors and ion channels, where the target cannot be presented as a soluble protein to the in vitro libraries and success with these types of targets is almost non-existent.

When using in vitro technologies, the output antibodies are invariably of low affinity, and there may be complex biological assays linked to the mechanism of action that such low-affinity antibodies cannot be reliably measured, and there is an elevated risk that if we take forward for optimisation, we will have limited biological activity or wrong mechanisms of action (allosteric vs competitive inhibitors etc). Here it is necessary to use in vivo because we will get to proof of concept in man (e.g., a cancer patient) in a more expedient manner thus benefiting patients sooner.

Why were they not suitable?

There is no suitable alternative to animal breeding.

Non-animal alternatives are suitable for scientific experiments to an extent, as explained above. However, GA mice must be used at the point where a whole animal is necessary to study the complex and multiple interactions between its genome and its phenotype; or to generate specific antibodies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Each breeding scheme is calculated based on the information from the strain, the genetic background, and the characteristics of the cohort required (age, genotype, sex). The numbers of animals required at each stage of the life of the colony (ie, maintenance, expansion, backing up) will be calculated to minimise the number of animals while safeguarding the quality of the colony over time.

The accuracy of the calculation is built on years of knowledge (predecessor from this Licence and expertise at the breeding centre), as well as the use of MCMS, a bespoke database originally created to support the recording of GA animal data.

Regular meetings between our teams will ensure that the requests for cohorts are timely and well defined. Lines of low use will only be bred when required and active mating will not be constantly maintained. Where possible, the line will be cryopreserved and removed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Breeding strategies are calculated considering several factors, including:

Strain information: fertility characteristics, complexity of the genetic construct and expected germline transmission. Binomial distribution statistics are used to assess viability of strains.

Characteristics of the experimental cohort required (genotype, sex, age, any phenotype where relevant; frequency of requirements).

Sample size calculations, determined at project level, in collaboration with the scientists.

The design of each experiment is discussed at the outset with members of the Scientific management team. The sample size and the justification for that are discussed, as well as how to generate the cohorts using the fewest mice possible. For example, if the cohort can be split into the batches that can be used more than 7 weeks apart, the breeding stock can be reduced by ~50%.

Regular meetings between our teams will ensure that the requests for cohorts are timely and well defined. Lines of low use will only be bred when required and active mating will

not be constantly maintained. Where the need for a line allows, the line will either be cryopreserved and removed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In cases where the viability or the phenotype of a line is unknown, a staged expansion breed will be carried out in the first instance to ascertain whether there are any unexpected welfare concerns.

For single allele breeds, tried and tested strategies can be used for the most efficient breeding. For more complex crosses, often several strategies are considered and assessed for efficiency in terms of timing, mouse numbers and welfare impact.

As part of initial characterisation of oncology models, cell lines and fragments determine whether transplantable models (e.g., models using tumour fragments) can be generated. This will reduce the requirement to specifically breed mice to support these experiments. Given the potential number of mice that are bred but not used during the generation of complex models, this has the potential to significantly reduce animal numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All aspects of the breeding protocols are refined in line with the 3Rs recommendations. For instance, the mice are housed in social groups, in environmentally enriched cages. The health status of the animal facility is tightly controlled through a stringent health monitoring programme, and all lines must be imported into the barrier of the facility through rederivation.

Care is taken to minimise disruption during daily checks of the cages, especially if young litters are present. Pups are checked for common health issues early and regularly, to prevent any inbreeding of an undesirable trait (e.g., malocclusion, alopecia). When required by the phenotype, weaning is done in coordination with genotyping, to avoid disrupting hierarchies and social groups later on.

All genotyping will be done on this licence using ear biopsy, which is also used as a method of identification. This should cause no more than minor and transient pain and prevent the need for any other procedure to be inflicted to the animals (except in <1% of cases where a second biopsy is needed).

Conventional breeding methods are used, and care is taken to ensure that the females are of appropriate weight/size.

Genetically altered mice will be bred in a way that causes the least harm to the animals. For example, if when carrying homozygous mutations, a mouse strain has a harmful phenotype, we will choose breeding techniques that minimise the number of pups born homozygous. It is not the scientific intention for any mice on this PPL to suffer from adverse effects.

Mice due to develop advert phenotypes will be moved on to PPL with the authority before the adverse effects become overt. In the case where these adverse effects are present before mice can be moved to another licence, or in breeding stock, specific justification will be detailed in the protocols.

Additionally, a large proportion of lines on this licence will be new or bred in specific combinations for the first time. In these cases, it is not possible to know what the adverse effects will be. Specific humane endpoints will be put in place based on an extrapolation of the symptoms in humans and predicted adverse effects in mice. For example, where humans have a neurological disorder with movement abnormalities, we might expect tremors or gait abnormalities in mice.

Why can’t you use animals that are less sentient?

Mice used on this licence must be of an age that is suitable for breeding; and their offspring need to be at an age suitable for shipping for use under other licences (usually, young adults).

Projects will be carried out on this licence when it is necessary to assess the gene function in a mammalian system. The ability to modify genomes in a complex way is more advanced in mice than in other species, although simple modifications are now possible across other species due to the advent of CRISPR/Cas9 technology. Additionally, and critically for genetic studies, there are a large variety of mouse inbred strains in which a genetic modification can be assessed in the context of a standardised genome. Such inbred strains are significantly rarer or not available for other species, including species traditionally considered as ‘less sentient’ such as zebrafish. Mice have a wealth of baseline data from which to draw comparisons with, both at a phenotype and genetic level.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Ear clipping is likely to be the only invasive procedure animals are subjected to during their life under this licence. Ear clipping will provide the biopsy tissue required for genotyping. The team performing these biopsies are highly experienced, and will carry the procedure quickly, minimising distress with excellent handling skills and skilful use of the ear notching device.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For terminal procedures routes and volumes for administration of substances are taken from LASA guidelines.

All procedures, including husbandry tasks, are carried out to strict standard operating procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PPL holder and managers working on this licence will attend national and international conferences focused on the 3Rs in animal science such as the 3Rs symposiums, NC3Rs, IAT, FELASA meetings, and meetings of the European College of Animal Welfare Science, Ethics and Law.

They will attend talks, give talks or run and/or take part in workshops focused on increasing reproducibility, reducing animal numbers and refining practices.

The PPL holder will also attend more specific disease-based conferences and undertake CPD through their employer's programmes to continue learning about the development of animal-free disease models.

Any near-miss or incident will be seen as an opportunity to improve, root causes will be identified, and any new refinement will be incorporated in working practices for the benefit of this PPL.

117. Understanding endogenous protective mechanisms in osteoarthritis; towards a new approach for disease management

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

osteoarthritis, disease-modifying drug, protective pathway

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to establish and utilise a mouse model of knee osteoarthritis (OA). The model will allow us to selectively knockout the gene encoding our protein of interest in the cartilage of adult mice in order to *define molecular and cellular pathways in joint tissues that protect against the development of OA*. This will help us to understand why some people suffer from symptomatic OA, whereas others do not, and will support our development of a new treatment for OA.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Osteoarthritis (OA) is a serious disease affecting 1 in 3 adults over the age of 45 in the UK. Pain and loss of mobility in joints (such as the knee) prevent people with OA from carrying out daily activities, which has a huge impact on quality of life. In addition, OA is associated with increased risk of heart disease, mental health problems and reduced life expectancy. There are no drugs disease-modifying drugs for OA and more than 160,000 joint replacements are performed each year in the UK to relieve pain and restore function. People with OA have identified a major need for treatments to reduce symptoms and delay joint replacement surgery. Previous research has developed our knowledge of how joint damage occurs in OA, but we don't know why some people develop symptomatic OA whilst others don't. Understanding the inherent molecular pathways that inhibit the OA disease process will pave the way for new treatments for this debilitating condition.

We have identified a protein that is made in joint tissues such as the cartilage, where it has protective effects and reduces inflammation. We are developing a drug for OA that is based on this protein and harnesses its beneficial activities. We have shown that our drug candidate can protect cartilage from damage and reduce pain in rodent models of OA. The focus of this project is to use a gene knockout mouse model to determine how the protein regulates molecular pathways joint tissues, e.g. following the type of injury that can progress to OA in humans. Informative studies on the early stages of OA development cannot be carried out with human tissues, due to a lack of availability of suitable samples.

The proposed work will support the development of our drug candidate, by providing information on its mechanism of action. Together with other ongoing work, where we are using tissue donated from joint replacement surgeries to explore pathways in established OA, this project will also help identify molecular markers to underpin the diagnosis and treatment of OA, including personalised medicine approaches.

What outputs do you think you will see at the end of this project?

The research outputs of this project will include:

A mouse model of osteoarthritis (OA) where the gene encoding our protein of interest is selectively knocked out in the cartilage of adult mice.

Data from our mouse model showing how our protein of interest influences the onset and progression of OA, both spontaneously and following surgical destabilisation of the knee joint. These will include imaging data and joint 'damage scores' to demonstrate whether OA develops more quickly and with greater severity in our model compared to controls, data on joint mobility and data on the expression of genes and proteins that are associated with pathways that promote or inhibit the onset and development of OA.

Insights into the mechanisms by which our protein of interest has protective effects in joint tissues and how our drug candidate harnesses these mechanisms in the treatment of OA.

We will publish our findings in open access peer-reviewed journals making them available to the OA- research community.

We will present our findings in the form or posters and oral presentations at conferences. The project will support our development of a disease-modifying treatment for OA.

Who or what will benefit from these outputs, and how?

These outputs have the potential to benefit patients with OA - a very common and debilitating condition - by improving our understanding of the disease process and, ultimately, by supporting the development of a new disease modifying treatment.

The outputs will also be of value to others who are conducting basic and clinical research in OA by providing new insights into the regulation of disease-associated mechanisms.

How will you look to maximise the outputs of this work?

We will maximise the outputs by:

Disseminating the outcomes of our work through conferences, seminars and publications, where we will publish the outcomes of both successful and unsuccessful approaches.

Continued collaboration with others in the OA field - e.g. by making our knock-out mouse model available to other researchers.

Working with our Faculty Media Relations team to disseminate outputs through the media.

Engagement with patients affected by OA and with clinicians to ensure that our work to develop a disease-modifying drug for OA is aligned with their priorities and needs.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will utilise mice as a model of human osteoarthritis.

Use of a mouse model will allow us to carry out genetic modification to generate a cartilage-specific gene knockout in order to specifically explore our protein of interest in the knee joint during the development of OA. The generation of the gene knockout will require a breeding programme, which will use pregnant mice, embryos and all life stages from birth to adult mice.

Cartilage-specific gene knockout will be stimulated in young adult mice at the point when they have a mature musculoskeletal system.

We will use these knockout mice to explore the spontaneous development of OA over a timeframe of up to 18 months.

We will also use the DMM (destabilisation of the medial meniscus) model of knee OA, where surgical destabilisation of the knee joint causes OA to develop over a few months. This model is used by many researchers in the OA field and is well accepted as providing informative data on disease-associated pathways and the effects of therapeutic

interventions. For example, we have previously tested our drug candidate in the DMM mouse model.

Typically, what will be done to an animal used in your project?

The generation of genetically modified mice will involve natural mating of animals. The mice will be kept in standard conditions for up to 15 months.

To induce cartilage-specific gene knockout, mice will be treated with tamoxifen. This will be administered either orally or by injection.

To induce OA, mice will undergo a precise surgical procedure (under anaesthetic) to destabilise the knee joint. Animals will be humanely killed at a series of endpoints after surgery (up to a maximum of 20-weeks) so that joint tissues can be examined for molecular and cellular changes.

Some mice will be kept for up to 18 months without joint destabilisation surgery and then humanely killed so that joint tissues can be examined for molecular and cellular changes.

Some mice will be treated by a series of injections (up to a maximum of 3 times per week) with our protein drug candidate.

As well as the endpoint examinations of knee joint tissues, the development of OA might also be examined using non-invasive imaging methods, such as x-ray and MRI (no more than once per month), and by observation of the animals' mobility (a maximum of once per week). Control mice, e.g. without gene knockout or with mock surgery, will be included for comparison in all elements of the project.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected as a result of the breeding of genetically modified mice.

Short-term treatment with Tamoxifen can result in some weight loss, hair loss and signs of urinary tract infection; these effects will be short-lived.

Following joint destabilisation surgery, mice might experience some inflammation, discomfort and ultimately arthritis in their joint - i.e. a swollen joint and possibly limping. Our previous work in the DMM model has shown that, although we can see changes in the joint tissues at the endpoints of experiments, mice show very little sign of discomfort.

Injection with our drug candidate is not expected to have any adverse effects, based on our previous work.

Expected severity categories and the 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 breeding programme is a mild procedure. No animals are expected to experience adverse effects.

The effects of tamoxifen treatment may be mild or moderate; this will be minimised by optimising the dose and route of delivery. Most animals will experience some effect.

The effects of DMM surgery may be mild or moderate. All or most animals will be affected.

The spontaneous development of OA in our gene knockout mice may be mild or moderate. We do not yet know the extent to which OA will develop in these mice.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The onset and progression of OA in humans is very variable. We consider that this is due to differences in intrinsic protective pathways within the joint. We have identified a protein that is made in the cartilage and inhibits OA-associated processes. Defining the molecular mechanisms involved and how they influence joint structure and function will improve our understanding of OA and support our development of a new disease-modifying drug.

The use of a mouse model is essential to achieve our aim. Mice have a musculoskeletal system that is similar to humans and are well established as informative models of OA. Mice are also the ideal species for genetic modification. The generation of a cartilage-specific gene knockout for our protein of interest in young adult mice will allow us to specifically define the effects of this protein within the knee joint.

Mice mature much more rapidly than humans, so we will be able to look at the age-associated development of OA in our model over a relatively short timeframe (up to 18 months). In addition, surgical destabilisation of the knee joint in mice causes rapid onset of OA and is a very well accepted model of post-traumatic OA in humans.

In these mouse models we will be able to use non-invasive methods to assess changes in joint mobility and structure during the study timeframes. At the study endpoints we will also be able to quantify joint damage and determine the expression of genes and proteins implicated in OA. In the joint destabilisation model we will be able to gather data for very early time points (from 6 hours after surgery) through to a maximum of 20 weeks. Evaluation of precisely defined time points, from the point of onset and throughout the progression of OA, could not be achieved using human tissues.

Which non-animal alternatives did you consider for use in this project?

We have considered using knee tissue samples from people with late-stage, symptomatic OA that are collected during joint replacement surgery. We have already used these to explore how our protein of interest influences the expression of other proteins known to be associated with cartilage damage and how our drug candidate acts to suppress cartilage breakdown.

We have considered using tissue samples from people who have suffered knee injuries or who have early-stage OA, for this project. We have also considered the use of cell culture models of cartilage, which we have used previously to generate mechanistic data.

Why were they not suitable?

The proposed work requires a model system that provides an accurate representation of the whole knee joint and where we can carry out analyses at precisely defined time points that represent OA onset through to late-stage disease. Human clinical samples are not suitable because they would not allow us to carry out genetic manipulation. Furthermore: (1) the population of patients having knee replacement surgery for symptomatic OA is very variable, (2) there are no good diagnostic markers for early OA, so it is impossible to identify patients at specific stages of the disease, and (3) patients are seen by clinical teams at very variable times following joint injuries. Therefore, it is not possible to carry out an informative study using human samples.

Cell culture models could provide the option of genetic manipulation to knockout the protein encoding our gene of interest. However, OA is a disease of the whole joint and disease progression depends on the mechanical loading of the joint, which can only be achieved in a live 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 have estimated that up to 4000 mice will be involved in our breeding programme that will generate the genetically modified mice to be used for cartilage-specific knockout of our gene of interest. This is based on: (1) the number of experiments we plan to use these mice for, and (2) the conservative assumption that each litter of mice we breed will include one mouse with the correct genotype.

We have estimated that up to 1000 of the mice that we generate in our breeding programme will be treated with the drug Tamoxifen to stimulate cartilage-specific gene knockout. We will use up to 400 of these mice for our studies on the spontaneous development of OA. In addition, we estimate that up to 450 of the mice we breed will be used in the DMM surgical model of OA; this will include up to 50 mice as sham-operated controls. This is based on: (1) the different experimental time points we will use (in order to explore the effects of our protein of interest throughout the early stages of OA development), and (2) previous work by ourselves and collaborators using the DMM model, which suggests that each experimental group will need to include 10-12 mice in order to generate informative data. Approximately 50 control mice that have not been genetically modified will be used to generate baseline data in the DMM model of surgically-induced OA.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the outcomes of work in our own and collaborators labs and taken the advice of a statistician to reduce the number of mice being used in each experimental group. We will use pilot studies to further adjust group sizes as the study progresses.

By using both male and female mice in the spontaneous OA and DMM-induced OA studies we will reduce the numbers of genetically modified mice that we need to breed. Furthermore, generating data in both male and female animals is important with regard to the translation of our findings towards human clinical studies, where OA is common in both women and men.

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 continually review the outcomes of experiments and use our data to optimise animal numbers as the project progresses. For example, we will conduct pilot studies to optimise the protocol for tamoxifen induction of cartilage-specific gene knockout and to determine the group sizes required in the OA model studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use a genetically modified mouse model to allow selective knockout of a gene in the cartilage of young adult mice. This model will be used to explore the effects of the gene deletion on the onset and progression of knee osteoarthritis (OA). We will determine whether OA develops spontaneously in the knockout mice over time (up to 18 months); in this case we anticipate that any OA- like symptoms will be mild and slowly progressing.

We will also initiate OA by surgical destabilisation of the knee joint in the DMM (destabilisation of the medial meniscus) mouse model. This is a very well characterised model, which we have used previously. We have observed that whilst mice develop OA-like damage to their cartilage, they exhibit little or no evidence of discomfort/pain over the timeframe of our proposed experiments.

Why can’t you use animals that are less sentient?

OA is a complex disease that involves the whole joint and is influenced by factors including joint mechanics as well as cellular and molecular processes. In order to explore the onset and development of human OA we need to use a live animal model with a mature musculoskeletal system that is similar to an adult human. The adult mouse fulfils this requirement. In addition there are well-established methods for genetic modification and for surgical induction of OA in mice - both of which are essential for the proposed project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The major welfare cost to the mice in this study is the development of OA-like symptoms. Since the purpose of this project is to study OA in the mouse knee joint, this welfare cost cannot be changed.

With regard to the Tamoxifen induction of cartilage-specific gene knockout in our mouse model, we will carry out pilot studies to determine the least harmful way of administering Tamoxifen, whilst still ensuring effective knockout (determined by genotyping). We will preferably use an oral method of administration. Tamoxifen treatment can cause some weight loss and signs of general malaise such as loss of appetite, hunched posture and piloerection, but these are typically transient.

With regard to the DMM surgical model of OA, we will continually monitor our procedures for pre- and post-operative care to ensure that these are optimised. For example, using sterile techniques to prevent infection at surgical sites and making adjustments to food, bedding and enrichment and using post- operative analgesics if required. In addition we will carry out pilot studies and order our experiments so that the data we gather can be used to refine the numbers of animals being used at each stage, i.e. to ensure that all experiments are informative, but without using more animals than necessary.

As well as collecting data on joint damage and gene expression profiles at the end of each experiment, we will use non-invasive methods (such as imaging and observation of animals' mobility) during the course of each experiment so as to maximise the information we obtain, but without any additional welfare costs to the mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use published documents recommended by NC3Rs to ensure that we are using the most refined approaches in all our experiments. With regard to DMM model of OA, we will regularly review the scientific literature for work by other researchers in the field to identify opportunities for refinement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are registered with NC3Rs and subscribe to their monthly newsletters, which will keep us informed regarding advances in the 3Rs. We will utilise the NCRs Experimental Design Assistant as well as seeking guidance from our local NVS and NACWO to inform the implementation of any advances that can be applied to our project.

118. Immunisation against infectious diseases

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

vaccine, influenza, antibody, therapy, pandemic

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to study and develop vaccines and therapeutic antibodies against influenza and other emerging pathogens.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 effective vaccines or therapeutics for influenza. Influenza epidemics have been causing up to 600,000 deaths annually and influenza pandemics cause a greater mortality. The current flu vaccines are based on prediction of which strains might be circulating in the coming years. On good years (in which the prediction is accurate) the effectiveness of the vaccine is ~70%, on bad years (like in 2013-2014 season) the effectiveness was only 10% due to a wrong prediction. Apart from influenza, as we learnt from the COVID-19 pandemic, it is important to study vaccine platform which could be used in the future as a pre-pandemic preparedness for emerging pathogens.

What outputs do you think you will see at the end of this project?

New vaccines against influenza and other emerging pathogens that are more “universal”. For instance, an influenza vaccine that would work against future strains without relying on prediction and therefore does not require annual update. Better understanding on the mechanism of protection mediated by vaccines against influenza and other emerging pathogens. We hope to eventually translate these vaccines for use in human.

Who or what will benefit from these outputs, and how?

We and the scientific community will be able to understand better on how vaccines against influenza and other emerging pathogens work, therefore able to design better vaccines in the future. This eventually will benefit the general populations when more effective vaccines are produced.

How will you look to maximise the outputs of this work?

We have established collaborations locally, within the UK and internationally in which we constantly share and discuss findings, experience and results from animal studies to help each other to improve the quality and outputs of research

Species and numbers of animals expected to be used

- Mice: 14,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We used adult mice because they are the lowest vertebrate group in the evolutionary tree for which the immune systems are well understood. Apart from that, we and other have extensively characterised influenza infection model in adult mice. Juvenile animals do not have a fully functional immune system therefore not a suitable model for our studies.

Typically, what will be done to an animal used in your project?

Typically, an animal will be immunised with a vaccine or a combination of vaccine (proteins, nucleic acid, viral vectors) and immune responses will be measured post immunisation in sera. Occasionally, immunised animal will be challenged with an infectious virus (eg. live influenza virus) and the protection of the vaccines against the infection will be studied. Adoptive transfer of sera, monoclonal antibodies, or cells will also be performed followed by challenge with an infectious virus. Occasionally, partial bone marrow deletion by irradiation or chemicals will be performed on mice to create bone marrow chimerea mice prior to immunisation. In certain experiments, in vivo live imaging (IVIS) will be used to study the biodistribution of vaccines/immunomodulators (tagged to bioluminescent proteins).

What are the expected impacts and/or adverse effects for the animals during your project?

(Protocol 2 Step 1) Mice undergone bone marrow ablation and rescue might experience transient weight loss (<15%) than don't typically last longer than 3 days.

(Protocol 2 Step 3) Mice that received transgene inducing or deleting agents might experience transient weight loss (<15%) and mind skin redness at the site of injection which typically improve within 24 hours.

(Protocol 2 Step 4) Mice receiving passive cells transfer might experience (extremely rare) embolism caused by clumping cells manifested as altered behaviour, hunching, laboured breathing and/or non- responsiveness.

(Protocol 2 Step 5) Mice receiving intranasal delivery of immunomodulators or vaccine might experience transient breathing difficulties that don't normally last longer than 30min, intramuscular injection might cause skin reddening at site of injection which normally improves within 24 hours. Mice that are immunised are expected to experience transient general malaise for up to 48 hours.

(Protocol 2 Step 6) In the influenza challenge model, unimmunised mice are expected to transiently lose weight over the course of 48 to 72h. Mice are monitored closely during this time and will be humanely killed if lost 20% of weight.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

70% Mild

15% Moderate

15% subthreshold

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The experiments described in this project license cannot be done using in vitro cell culture or isolated organs of animals as they often involve several cell types of the immune system and the complex interactions with each others. These interactions cannot be mimicked in vitro. In addition, the use of genetically modified mice allows us to study the effect of a single gene on the immune system, which cannot be studied in vitro. The mouse influenza challenge model and the immune responses have been well established and well understood.

Which non-animal alternatives did you consider for use in this project?

In vitro assays.

Why were they not suitable?

In vitro assays are not able to help us to answer most of the scientific questions as they cannot reliable mimic the multicellular environment and the complex immune system that determine the action of the vaccines or treatments (immunomodulators, monoclonal antibodies or sera).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 use of in vitro methods limits the numbers of animals required for the in vivo research. Prior to an in vivo experiment we consult the literature and try to minimise variables in the experimental design. Where necessary we seek advice from people who performed such experiments and experts in the relevant techniques who can advise us on the expected outcomes, unusual controls that may be required and adverse effect that they have encountered. Review of the previous experiments have confirmed that 6 animals are required to determine the effect of a vaccine or to compare multiple dose/type of vaccine. We run pilot experiments with limited size groups to determine intra and inter group variations and address simple questions before expanding our study further. After the pilot experiments, usually with groups of 3-4 mice, and if no adverse effects are detected, either additional repeats are performed with equally small group-size until the required statistical significance is achieved or larger group size are used based on the variation of data from the pilot experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whenever appropriate, the NC3R’s EDA will be used to aid experimental design especially in determining the appropriate sample size and the ARRIVE guideline will be followed. We also perform in vitro screening experiments whenever possible and suitable to screen immunomodulators/monoclonal antibodies which allow us to narrow down the concentration range that is required to be tested in animals, therefore reducing the number of groups of mice. In experiment involving adoptive T cell transfer, we continuously improve tissue processing techniques to increase the yield of cells harvested, therefore less donor mice will be needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will ensure efficient breeding of colony and whenever available we will share data and resources (e.g. tissue) with other research groups. We will use refined statistical analysis and computer modelling (whenever appropriate), and regularly re-examining the findings

of previous studies al (systematic review) to help optimise the number of animals in subsequent experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using mice because mice are the lowest vertebrate group in the evolutionary tree for which they have a functional immune system that can be used to understand the immune responses in humans. As our background strains, we use both genetically altered and wild type mice for which much groundwork has been obtained and many genetically altered derivatives generated. This allows us to hone in on our specific area of interest with an understanding of the contextual settings.

Our understanding of the infectious is continuously increasing and we can anticipate much of the developments allowing us to identify variations at early time points. Typically, mice will be immunised with a vaccine (single or multiple doses, typically intramuscular but also sometimes via intranasal, intraperitoneal or intravenous route) and sera and/or organs (such as spleen and lymph nodes) will be harvested to measure the immune responses elicited by the vaccine. Occasionally, for influenza vaccine, immunised mice will be infected with a live influenza vaccine to study the protection efficacy. These are the most refined method as the various routes of administration are not expected to cause any suffering that’s beyond mild. From our past experience characterising the kinetics of the immune response, we are able to minimise the number of blood sampling steps such that blood is taken at the most informative time points post-immunisation and the blood sampling via tail vein is minimally invasive. The combinations of mouse strains and influenza virus strains/doses typically used are well characterised and recovery is expected in wild-type mice; although these infections will in most cases be symptomatic, adverse effects are typically transient. Infection resolution is expected, even in mice which exceed 15% weight loss, as discussed below. It is important to use a 20% weight loss limit since Influenza virus infected mice frequently lose 15% weight yet go on to make a full recovery; imposing a humane endpoint at 15% weight loss for influenza infection would mean that we might be less able to discriminate between mice developing an immune response that will clear the viral infection and those mice that are not protected.
Mice in which an effective immune response is mounted are not expected to exceed 20% weight loss. For genetically modified mice, breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in house database and will be used to make decisions on future breeding animals and also to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

Why can’t you use animals that are less sentient?

Mice are the lowest vertebrate group in the evolutionary tree with a fully functional immune system that can be used to study the immune responses in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be monitored regularly post procedures, and increased monitoring will be done when a new vaccine/adjuvant/treatment or procedures are administered. Analgesic will be used to minimise or remove pain post procedures accordingly. Cup or tunnel handling will be used when handling the mice to reduce stress imposed to the mice. Mice will be housed in group and in appropriate number to ensure that they have enough space to perform normal social behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to the LASA, PREPARE and ARRIVE guidelines for reporting these studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through frequent review of the NC3R in the UK and CAAT (Center for Alternatives to Animal Testing) I will keep informed about advances in the 3Rs and having access to the regional 3R’s manager and the Named Information Officers.

119. Analysis of Pain Pathways

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pain, Sensory Neurons, Nociception, Analgesia, Gene therapy

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to define mechanisms important in pain, particularly chronic pain in mouse models of human pain conditions. Importantly, all human analgesic drugs work well in mice. Although animal models have not always been useful in testing potential new human analgesics, there is a strong rationale for their use particularly in conjunction with human pain genetics which gives insights into potential targets that can be explored in mice.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The Global Burden of Disease Study 2016 found pain and pain-related diseases, particularly low back pain and migraine, are the leading causes of disability and disease burden globally. Low back pain and migraine are responsible for 57.6 million and 45.1 million years of life lost due to disability, respectively. This means the burden of pain conditions is higher than that due to depression, cancers, diabetes and Alzheimer’s

disease. In Europe alone, almost one in five individuals or 20% of the adult population report having moderate or severe chronic pain. This means there are 150 million people in Europe experiencing chronic pain. Chronic pain has been linked to numerous physical and mental conditions and contributes to high health care costs, early retirement and productivity lost. Clearly, there is a need to investigate the mechanisms that initiate and propagate chronic pain and to identify new analgesic targets to better treat the substantial number of people affected by the condition.

What outputs do you think you will see at the end of this project?

Our program of work is devoted to unraveling the cell and molecular mechanisms that contribute to different types of pain, to devise new approaches focusing on novel molecular targets that will be revealed by these studies.

Who or what will benefit from these outputs, and how?

As 7% of the population suffer from severe pain, the utility of identifying new therapeutic targets for pain is self-evident. There is a huge unmet clinical need for improved and targeted analgesia, as many chronic pain conditions are addressed with similar classes of centrally-acting drugs that do not differentiate between distinct diseases and classification of pain states. Our experimental approaches to define distinct cellular and molecular targets in different pain conditions is essential to providing candidate drug targets for more effective pain relief. Our experiments will define new analgesic targets within the lifetime of the licence. Although, drug development can require considerable time due to regulatory constraints, but the potential reward for pain relief remains very high.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work by having a range of relevant available mice for genetic studies and collaborations with pain groups around the world. These tools will allow us to assign particular subsets of neurons to precise functional roles in models of different pain syndromes, through electrophysiological and behavioural analyses. Our approach to exploit *in vivo* assays in animal models of chronic pain is fundamental to acquiring insight of the nervous system organisation for nociceptive processing – both at the molecular level that we can manipulate and at the whole system level that we can monitor. Moreover, the mice that we generate and characterise will be invaluable for use by other researchers and the Pharma industry to examine pain mechanisms. Composition of matter patents have been filed on analgesic targets that we have identified using mouse genetics.

The benefits that we will gain will be in the short term:

Provision of a set of research tools for pain studies of use to the whole world pain research community. The benefits that we will gain will be in the medium term: New insights into the cellular mechanisms that underpin different pain syndromes in rodents. The benefits in the long term will be: Identification of key genes that cause distinct pain syndromes and that present new targets for the development of therapeutic drugs.

Species and numbers of animals expected to be used

- Mice: 20900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used for this project as they represent the most appropriate genetically amenable species for this type of work; they are similar to humans genetically, and physiological systems for pain are largely similar and all forms of analgesia used in humans are also effective in mice. Many key physiological systems and structures involved in pain processing were originally identified in rodent and other models and validated in more recent human studies.

On top of this, decades of research have resulted in highly advanced and efficient techniques developed for investigating neuroscience questions in the mouse, such as optogenetic studies to control the activity of cells by light. Importantly, the mouse is highly amenable to genetic modification, allowing transgenic identification of specific cell types crucial to the fulfilment of the project.

Over several years of pain research, we have been able to establish protocols to minimise stress in rodents to ensure the best welfare of all animals and maximum scientific output from all studies requiring consistent animal handling. We have also developed new techniques to replace conscious animals with unconscious animals (e.g., in imaging experiments to assess cell activity) to reduce suffering.

Typically, what will be done to an animal used in your project?

The first two sections within this licence are for breeding and maintenance of the mice. The first section is a mild protocol where no harm should occur. The second is moderate, as some of the genetically altered animals may experience alterations to pain sensation that should be monitored closely for injuries or potential suffering.

The third section is mild, involving a range of behavioural tests for mutant mice, to establish whether they have loss or gain of pain. These produce data on the pain status of the mice. There are several sections described as moderate protocols that mimic human pain conditions. Use of these in animal models is necessary for understanding distinct mechanisms in different pain states that may be treated with different types of pain killers.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of our research involves procedures of mild severity using the majority of the animals. There are exceptions which we have identified as falling into the moderate classification for the protection of the animals. Expected adverse effects might include post-operative stress or discomfort, but these will be quickly identified, and measures taken to minimize suffering. In all these cases, or when unexpected clinical signs appear, we will consult our NACWO and NVS. At the end of each procedure, animals will be euthanized according to certified Schedule 1 procedure and tissues will be isolated for further 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 animal type)?

All our protocols are conducted upon mice and consist of either sub-threshold mild or moderate procedures.

Our breeding and maintenance protocols consist of a mild protocol (90% of animals) where no harm should occur, and also a moderate breeding protocol (10% of animals, for animals that have some pathology).

The majority of our animals will be studied using mild protocols (90%) with a few taken to sub-threshold non-recovery imaging experiments directly (10%). These mice are studied under terminal anesthesia

We have several moderate protocols which mimic human chronic pain conditions and protocols involving induction of genes or application of gene therapy agents that require cautionary restrictions to protect the animal's welfare.

Inflammatory pain 20%
neuropathic pain 20%
cancer pain 20%
musculoskeletal pain 10%

What will happen to animals at the end of this project?

- 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?

Pain is arguably the biggest clinical challenge of the age and is increasing with aging populations. The hope that human studies using functional imaging could be used to study human pain mechanisms has been heavily criticised after the demonstration that the so-called pain matrix is not directly related to pain perception, and only self-reporting of pain intensity from humans is a useful guide to drug efficacy. We apply many well-established protocols that have been refined over decades of use.

The use of animal models of acute and chronic pain conditions is fundamental to providing insight into molecular, cellular, and systems organisation of damage sensation that cannot be determined in humans. Such animal models offer the ability to produce fine-tuned characterisation of neurochemistry and anatomy, alongside the benefits of excellent temporal and spatial resolution with imaging and electrophysiological recordings of cellular activity. A very important finding from our recent studies has demonstrated that sensory neurons *in vivo* have properties that are lost when the neurons are excised and studied *in vitro*. This means that studies of sensory neurons in culture need to be interpreted with great caution, as the *in vivo* situation is so clearly different from culture conditions.

Which non-animal alternatives did you consider for use in this project?

Where appropriate we conduct in-depth genetic analysis of potential targets of interest prior to animal work using gene sequencing technologies and molecular and electrophysiological methodologies. Human genetic insights are also valuable.

In vitro and cell culture-based alternatives have been, and will be, used by us as replacements wherever possible (for example electrophysiological studies of channel function) to examine selected aspects of the responses we aim to more fully decipher *in vivo.*

Why were they not suitable?

Whilst we fully acknowledge the strengths of *in vitro* and cell culture-based alternatives and have reviewed their use, we are aware and appreciate their limitations. As valuable as these studies may be, they can never tell you which physiological systems are critical for pain perception – only behavioural studies can tell us this. The amount of information we can obtain from carefully designed *in vivo* studies is actually greater and more valuable in translating to clinical studies in its impact than *in vitro* studies that may superficially appear to be more attractive in terms of minimising animal use and discomfort.
The study of pain pathways thus requires the analysis of pain behaviour in animal models.

With our recent findings (PMID: 27847865), we demonstrate that sensory neurons *in vivo* have properties that are lost when the neurons are excised this finding unfortunately means that many *in vitro* studies designed to help with the 3Rs actually give us misleading data, re-inforcing the need for whole animal studies at the moment.

In a similar fashion, *in silico* techniques have no obvious utility when the fundamental mechanisms of pain pathways are so very poorly understood.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experimental designs – unbiased and adequately powered studies – are applied in order to identify the minimum number of animals we need to use and identify the best technical approaches we can employ to answer the specific question being posed. We have extensive experience in animal pain behavioural studies and have found that for the paradigms described in protocols 3-8 we can obtain statistically meaningful data with groups of 6-10 animals (PMID: 32663221).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will optimise our breeding strategies to produce minimal numbers of animals. For example, we will breed homozygous floxed mice with homozygous floxed mice that

express one copy of a Cre- recombinase, so that all the progeny of the mating will either be experimental animals (i.e., genes will be deleted) or appropriate controls (i.e., floxed genes will be present but not deleted). It is important to emphasise that gene deletion studies are more reliable and reproducible than gene knock-down studies using antisense oligonucleotide or siRNA approaches (PMIDs:22531176, 22188729, 18669863, 16850455, 15621361).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In several of our protocols, the cumulative severity is low enough that the same mouse can be maintained for continued use on different days, for measuring thermal and mechanical hyperalgesia. This action reduces by half the number of necessary mice. Group sizes in all other studies will be in accord with power analysis applied to previously generated data or published findings from our own or other groups (PMIDs: 33823138, 33693512, 31911151, 30638309, 35233469, 29394316). Wherever it is possible we will also exploit contralateral limbs as controls in order to reduce the numbers of animals required still further; the possibility of exploiting such controls is another area in which future reduction in numbers may be achieved. This may not always be possible, however, but efforts will be made in all initial investigations to secure the validity of internal control samples. We also use the help of on-line tools such as the ARRIVE guidelines form: a guideline basis for the determination of experimental design at https://arriveguidelines.org/.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen to focus particularly on transgenic mice. This decision has been made as it will provide us with the potential to explore the role of specific genes in the response we identify, through the use of mutant and transgenic mouse models. It is possible to generate transgenic mice much more easily than other rodents.

Animal suffering will be limited in our studies by our strict monitoring of animals with regards to severity limits of associated protocols. Our use of protocols has been carefully designed to minimise trauma and suffering. We have chosen a range of models to cover different types of pain conditions as it is clear from previous research that there is not one common pathway for all types of pain. The number of different models allows us to look specifically at a type of pain and reach our objectives efficiently and by using as few animals as possible.

Neuropathic and bone cancer pain models involving surgery or strong chemicals are currently the only way to model neuropathic and bone cancer pain states in animals, and we will endeavour to review our methods along with the current literature to ensure that we are causing the least amount of suffering to the animals, in order to reach our objectives.

With all models used, we will minimise suffering by administering pain relief where possible. The animals will be culled immediately after the last time point of testing or as soon as social behaviour, grooming, weight loss/gain and/or wound healing indicate the animal is suffering to a level noted in the licence.

Why can't you use animals that are less sentient?

Mice will be used for this protocol as they represent the most appropriate species for this type of work; they are very similar to humans as in their genetic and physiological systems for pain are largely similar with all types of analgesia known to work in humans also being effective in mice. Many key physiological systems and structures involved in pain processing were originally identified in rodent and other models and validated in more recent human studies. On top of this, decades of research have resulted in highly advanced and efficient techniques developed for investigating neuroscience questions in the mouse. Importantly, the mouse is highly amenable to genetic modification, crucial to the fulfilment of the project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We routinely examine the scientific literature for advances in procedures. The majority of these also produce refinements in the experience for the animal and betterment of its welfare. A relaxed healthy animal produces far better outcomes for our research. Over many years of pain research, we have been able to establish protocols to minimise stress in rodents to ensure the best welfare of all animals and maximum scientific output from all studies requiring consistent animal handling. We have also developed new techniques to replace conscious animals with unconscious animals (e.g., in imaging experiments to assess cell activity) to reduce suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We consult trusted sources e.g., Handbook of laboratory animal management and welfare' by Wolfensohn and Lloyd (2003), The Laboratory Animal Science Association (https://www.lasa.co.uk) alongside the NVS.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Alongside regular scientific publications, we receive regular updates regarding the interesting advances in the 3Rs from our biological services team. Most of our researchers are registered with the NC3R's website (https://nc3rs.org.uk) for the latest developments in the field.

120. Understanding the link between stress-induced peripheral dysfunction and development of mental illnesses

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stress, Gastrointestinal tract, Neuropsychiatric disorders, Neurotransmitter, Brain function

Animal types	Life stages
Mice	adult, juvenile
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main aim is to better understand how exposure to negative environmental factors, especially psychosocial stress, and peripheral pathologies can engage the central nervous system and lead to development of mental health 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?

Neuropsychiatric conditions present a major burden for the sufferer and for the society. Depression and schizophrenia affect up to 27% of people during their lifetime. Attention deficit hyperactivity disorder (ADHD) affects up to 3.3 million children in the EU. Neurodegenerative disease such as Parkinson’s and Alzheimer disease affect a growing number of people with population ageing. An even larger number of individuals suffer from psychosomatic conditions, likely associated with stress and depressive symptoms, such as irritable bowel syndromes (IBS). Treatments for these conditions are often only partially

effective, and their mechanism of action poorly understood, with unpleasant long- term side effects.

Environmental factors modulated by stress, diet, metabolism acting on our gastro-intestinal systems can strongly interact with our central nervous system and contribute to the development of neuropsychiatric disorders. A highway of nerves runs from our brain to our digestive system, and messages flow in both directions between the two. This bidirectional regulation has not sufficiently been considered in relation to our understanding on the physiopathology and treatment of mental disorders. Indeed, environmental factors, including psychological stress, can cause major dysfunction in our gastrointestinal tract which in turn, via activation of a number of neural, endocrine, and immune pathways, has ability to change brain neuronal activity, central receptor sensitivity, cognition, response to stress, risk of drug abuse, and pain perception (nociception).

The project intends to explore mechanisms by which our gastrointestinal tract can react to environmental factors such as stress, diet and metabolism to transmit important changes in brain neurotransmission processes involved in neuropsychiatric disorders and to examine if this could have therapeutic implications.

What outputs do you think you will see at the end of this project?

The data obtained will be disseminated among the international scientific community through publications and presentations. The data generated will help to better understand gastrointestinal mechanisms modulated by stress and contributing to the development of neuropsychiatric disorders. Our data can also reveal new information about the role of diet and gut microflora on brain function, particularly on cognition and nociception. They may encourage the general public to modify their diet (for example with pre/probiotic compounds) to benefit their mental health. This may have a significant positive impact on public health in the sense of managing debilitating problems such as cognitive decline and visceral pain, which are extremely common. Cognitive decline is now a major health problem with our ageing population. The study would also provide more insights in our general understanding of the regulation of brain function by the intestinal tract and may demonstrate that manipulating diet and metabolism may improve the effectiveness of psychotropic drugs. Our strong long-standing connection with the industrial word, through project collaboration, will also contribute to the discovery of new product or new food formulation that can alleviate dysfunctions of the gut-brain connections and improve therapeutics.

Who or what will benefit from these outputs, and how?

During the course of the project we will ensure that our data will be disseminated through the scientific community by writing scientific publications and presenting our results to scientific conferences, which, in turn, will build up and establish connections and collaborations with other scientists and stakeholders.

Both scientific and clinical investigators should find our data of interest. We will also ensure that our publications will be accessible to the general public. This can be done through our University network (e.g. University Research repository) and/or educational and charity organisations.

How will you look to maximise the outputs of this work?

Collaboration with other Universities and with industrial partners will be a priority. During the previous project we have built up important collaboration on project looking at the

effect of selective diet on cognitive function and brain neurotransmission. In addition, our previous collaboration with industry has also contributed to demonstrate potential therapeutic effects of some products for psychosomatic and inflammatory gut disorders. It is our intention to pursue these collaborations.

During the course of the project we will ensure that our data will be disseminated through the scientific community by writing scientific publications and presenting our results to relevant scientific conferences, including our "negative" results, when we have not been able to verify our hypothesis.

Species and numbers of animals expected to be used

- Mice: 1250
- Rats: 950

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To investigate brain circuitry at the molecular levels involved in complex behaviour, we need animal models. The use of peripheral tissues from patients or volunteers, such as blood, has limited value. Some of the limitations have been overcome by using postmortem brain samples from human subjects; however, their availability is scarce and is often associated with confounding variables.

Animal research has made an enormous contribution to human health and welfare. Rats and mice have been the leading model organisms used in biomedical and behavioural research. Our experiments will be carried out on small rodents (rats or mice) which are the most suitable species for the proposed studies: 1) they are most convenient lab animals as they are small, easy to house and maintain, adapt well to new environment and reproduce quickly; 2) most cost effective species; 3) almost genetically identical in the same sex as they are mostly inbred to ensure more uniform results; 4) share virtually the same set of genes as humans which enables investigation of functional mechanisms, both at the molecular and behavioural levels. Exposing rodents to acute or prolonged stress has proven to be useful in understanding the molecular mechanisms underlying affective-like disorders. Such research cannot be carried out on living organisms with a too substantial genetic distance with humans as it requires investigation on functional mechanisms, both at the molecular and behavioural levels, which would be too different. Rats and mice are the animals the lowest in evolution on which such type of research can be carried out. Although other animals, such zebrafish, fruit flies and roundworms, can also be used, they won’t be suitable in this type of research as the organisation of their central nervous and digestive system is too different from human being.

Typically, what will be done to an animal used in your project?

Animals may be subjected to a specific diet or to a specific drug administered in the food or via the drinking water or via injection or gavage.

Animals may be subjected to some psychological and social stress tests such as the acute or chronic restrained test or the social defeat test (SDT). In the restrained test, animals are immobilised for a short period (1-2 hrs) no more than once a day for 1-10 days. In the SDT, tested animals are repeatedly subjected to bouts of social defeat by a more socially dominant cage mate for short periods (2-5 min) no more than once a day for 1-10 days.

Animals may be subjected to some non-regulated behavioural procedures, not expected to induce any significant stress, which will test whether a specific treatment or condition has not induced any behavioural change (e.g. open field tests, sucrose preference tests, motor function tests, cognition tests.

Animal may be anaesthetised for a short period for implantation of a subcutaneous device used for chronic treatment.

Animals may be administered chemicals that can induce intestinal irritation.

Animals will be terminally anaesthetised and will undergo some surgery for brain and intestinal investigations.

What are the expected impacts and/or adverse effects for the animals during your project?

Stress can induce mild weight loss (around 10%), social defeat tests can occasionally cause bite scars that typically resolve in a matter of hours. Stress can induce anxiety and depression-like behaviour that can be assessed using specific (non-regulated) test, like the open field or the sucrose preference tests.

Most of the treatment administered will not normally cause more effects than minor discomfort, for example skin irritation at the site of injection.

Some chemicals can cause intestinal discomfort that can induce weight loss and gastrointestinal pain and dysfunction, including diarrhoea occasionally with some small amount of blood in the stools. These symptoms are mainly observed during the treatment period (maximum 7 days) and often progressively attenuate after the cessation of the treatment. Animals are carefully monitored to make sure these symptoms do not worsen.

Short anaesthesia should only induce minor discomfort during the recovery 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 animal type)?

We expected the severity to be mild for 80-90% of all our animals (rats and mice) and moderate for 10- 20% of them.
What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animal models is crucial for the determination of long-term effects of treatments. The determination of the neuronal changes induced by psychotropic medication and metabolic factors requires the presence of neurones in an intact brain. Neurons to be examined are within a particular brain structure which is regulated by the different inputs from other brain regions. The entire central nervous system is required for such study. Whenever possible we propose to use in vitro techniques. However, in in vitro conditions cells are not in their natural environment and are dissociated from neuronal connections. It is precisely these various inputs which are of particular interest in our studies.

Which non-animal alternatives did you consider for use in this project?

There is very little animal alternative for use in this project. Whenever possible we will consider using tissue culture from immortalised cell lines to perform some of our experiments on protein expression and release of mediators. However, this can be only limited to some investigation aiming to better understand intracellular pathways, while most of our experiments require the integrity of the tissues in an entire body .

However, whenever possible we propose to use brain slices taken from wild-type and transgenic animals killed by Schedule 1 to investigate the effect of drugs in vitro on brain cell electrical activity.

The use of peripheral tissues, such as blood, or from postmortem brain or intestinal samples from patients or volunteers could also be considered.

Likewise, we intend to perform in vitro neurotransmitter release experiments using brain slices or synaptosomes wherever possible, although similar limitations apply for this technique.

Why were they not suitable?

The determination of the neuronal changes induced by drugs requires the presence of neurons in an intact brain. Using tissue culture from immortalized cells can be only limited to some investigations as our experiments require the integrity of the tissues in the entire body.

Cells within slices are not in their natural environment and are dissociated from neuronal connections with other brain structures. Neurones to be examined are within a particular brain structure which is regulated by the different inputs from other brain regions. The entire central nervous system is required for such study. Whenever possible we propose to use in vitro techniques. However, in in vitro conditions cells are not in their natural environment and are dissociated from neuronal connections. It is precisely these various inputs which are of particular interest in our studies. In addition, the natural spontaneous activity of some neurones may be lost within a slice preparation; this requires additional manipulation of the preparation (eg adding an excitatory substance in perfusion medium) which may impair the validity of the data.

Availability of post-mortem brain tissue is scarce and is often associated with confounding variables. The use of tissue culture to investigate consequence of stress on brain activity is limited.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A large number of animals will be used for protocol involving terminal anaesthesia only.

Numbers are calculated to meet the requirement for statistical power. For pilot studies and trial experiments, numbers are estimated from our previous experience, the number of primary and secondary objectives and investigators involved in this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will follow step by step the approach provided by Experimental Design Assistant to design our various protocols. We will perform the power analysis to calculate the number of animals needed for each experiment to ensure minimum number of animals will be used with acceptable statistical power.

Our protocols are internally reviewed by experimented scientists using an online submission system.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animal numbers will be minimised by optimising our experimental design. We use specific statistical analysis to identify the optimum least number of animals required under each protocol and this will be constantly reviewed.

Animal numbers will be minimised by optimising our experimental design. We use statistical power analysis tools such as G*Power-specific statistical analysis to identify the optimum least number of animals required under each protocol without compromising statistical power, and this will be constantly reviewed.
The number of animals estimated for use under each protocol will be calculated as the minimum number needed to meet the objectives and will be reviewed throughout the duration of this programme. In general group sizes of 5-6 are appropriate for electrophysiological and microdialysis studies and group of 4-6 are appropriate for ex vivo experiments and 6-10 for behavioural experiments.

We will optimise our surgical methodology and minimise the loss of animals by employing best practice at all stages following discussion with our NVS, for example by using good surgical technique, the use of suitable short acting anaesthetics, minimal surgery time, thermoregulation, aseptic procedures and appropriate postoperative care and analgesia.

We will also provide sufficient training to all investigators to ensure they can perform competently and consistently to avoid repeating experiments or needing more animals.

Wherever possible, in vitro studies will be used to obtain data.

Whenever possible, negative results will be disseminated to avoid other groups repeating the same experiments in order to reduce animal use.

We will use appropriate controls to prevent false results: for example use drug vehicles, inactive drug of similar chemical structure, and pharmacological blockade to confirm specificity of action. Rather than repeating controls, we will use "rolling controls" for each similar set of experiments spread over a period of time. We will optimise our surgical methodology and minimise the loss of animals by employing best practice at all stages following discussion with our NVS, for example use of short acting anaesthetics, minimal surgery time, thermoregulation, aseptic procedures and appropriate postoperative care and analgesia.

When appropriate we will design pilot experiments (2-3 animals) before embarking on full-scale studies, and carefully check literature updates to avoid replication of experiments already published. Wherever possible, in vitro studies using tissue culture sources will be used to obtain data that would otherwise be achieved using animal tissue sources, for example to estimate the affinity profile of a drug at specific target proteins.

The protocols used in all animal studies will be constantly reviewed for improvements in terms of minimising trauma and careful monitoring of the animals' condition by the personal licence holders and NACWO.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 carry our experiments on rodent (mice or rats) models.
To achieve our objectives, it is necessary to model or investigate complete neural pathways of the brain and this is not yet possible using in vitro models or cell cultures. To minimise animal suffering, our in vivo measurements of neurotransmitter function and neuronal electrical activity will predominantly utilise anaesthetised, non-recovery preparations rather than recovery preparations.

Why can't you use animals that are less sentient?

Our experiments will be carried out on small rodents (rats or mice) for the reasons we stated in the "Project Harms" section above. Rats and mice are the least complex species in which such studies can be successfully undertaken. Although other animals, such zebrafish, fruit flies and roundworms, can also be used, they won't be suitable in this type

of research as the organisation of their central nervous and digestive system is too different from human being, and therefore their functions of brain and digestive system and behaviour, as well as the underlying mechanisms, are different from humans. It would have little translational potential if these less sentient animals were used. As indicated above, our in vivo measurements of neurotransmitter function and neuronal electrical activity, which ends up each of our 5 protocols will only utilise anaesthetised, non-recovery preparations.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The principle of refinement is to minimise pain and suffering and improve welfare of animals. We will apply the following rules:
Routine health monitoring using a score-sheet to detect and intervene early in signs of ill health. The frequency of monitoring will be increased in the small number of instances when the likely incidence of adverse effects is uncertain (eg. novel drugs, previously unstudied lesion sites – see below).

Use of literature and other databases to aid selection of drugs and treatment regimens that produce optimal pharmacological effects and minimal adverse effects. Drug-induced adverse effects will be detrimental to the experiments and drugs with this potential will be avoided.

Use of literature and other databases to aid selection of neurotoxin and lesion sites which do not alter the animals ability to perform normal behaviours (movement, eating and drinking, cleaning etc.) and avoid impairment of sight, hearing or smell. To maximise the value of the experimental outcome and minimise the probability of adverse effects, neurotoxins will be selected on the basis of high chemical specificity.

Use of pilot experiments (2-3 animals) before embarking on full-scale studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will particularly follow advices from the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines which are intended to improve the reporting of research using animals – maximising information published and minimising unnecessary studies. The guideline, originally published in 2010, is constantly revised (last version 2020: Percie du Sert N et al. (2020). Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol 18(7))

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will utilise on-line resources including those provided by the NC3Rs, (https://nc3rs.org.uk/who-we- are/3rs which includes a resource library specifically for this purpose, https://nc3rs.org.uk/3rs- resources). By subscribing to NC3Rs newsletters, we will also get the latest updates directly.

We will also regularly attend to Training and Information Forum and are following up information provided by the ASPEL website.

121. Normal Blood Products

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Diagnostics, Plasma, Serum, Fibrinogen, Blood Products

Animal types	Life stages
Goats	adult
Sheep	adult
Alpacas	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?

Sheep, goat and alpaca blood donations will be taken and the:

blood products used in production of diagnostic kits

blood cells will be used to produce recombinantantibodies

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 have access to various blood proteins, cells, plasma, serum and whole blood that are used in manufacture and development of diagnostic kits. Recombinant antibodies can also be produced using extracted blood cells.

What outputs do you think you will see at the end of this project?

Expected outputs are that sera, plasma, blood or PBLs/ PBMCs are supplied as specified by the customer. Blood products have a wide range of uses and demands can fluctuate depending on customer requirements. Examples are:

Plasma supply to one of our customers is expected to be 300L over the 5 years of the project to meet production demands of a diagnostic kit

Creation of naive sheep and alpaca antibody libraries that will enable future antibody production without the constant requirement for animal use.

To supply whole blood for blood agar plates for diagnostic microbiology.

Who or what will benefit from these outputs, and how?

There is a continuing commercial demand for normal animal sera, plasma and blood, as reagents in immunoassays and other clinical assays. The majority of this is used to extract clotting factors for use as critical reagents in clinical chemistry assays, such as fibrinogen which is used directly in diagnostic tests for the monitoring of blood clotting function in patients taking oral anticoagulant drugs. Wherever in the world clinical microbiological investigations are carried out, the characteristic appearance of bacterial colonies on agar plates, supplemented with sheep blood is a universally accepted and recognised first stage of the isolation and identification process.

Whole animal blood can be used to test mechanical devices such as mechanical hearts as it provides a more real to life comparison than with artificial blood.

PBL and/or PBMCs from non-immunised animals are increasingly used to develop naive antibody libraries. These are extracted from the blood of donor animals.

How will you look to maximise the outputs of this work?

Use of larger animals such as sheep aim to maximise the outputs of this work by producing larger volumes from minimal numbers of animals. The production of blood products will be to demand to fulfill requirements without the need to produce excess product. Blood products usually have a very long shelf life which maximises the length of use without the need to replace products.

Dissemination of new processes and products throughout the company and our customer base will maximise the outputs as this will lead to better use of blood products in diagnostic kit manufacture and for better production of recombinant antibodies for use in diagnostic kits and therapeutics.

Species and numbers of animals expected to be used

- Goats: 5

- Sheep: 250
- Camelids: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Sheep, goats and alpacas are large animals and enable larger volumes of blood to be collected at any given donation. This leads to the requirement for lower numbers of animals to obtain a given volume of blood.

Adult animals are used as they are mature and better adapted to regular blood donation.

Sheep and goats, with their contractile spleens can adapt to regular bleeding, with no effect on their health.

Alpacas produce nanobodies and can have advantages over other large animal antibodies when used in therapeutic and diagnostic applications. Peripheral blood lymphocytes taken from healthy adult non-immunised animal blood can be used to create naive nanobody libraries by phage display thus reducing the number of animals required to produce nanobodies.

Typically, what will be done to an animal used in your project?

Typically, an animal will have a set volume of blood collected, correlating to its size and species. This will involve insertion of a needle into a vein and collection of blood into a blood bag, comparable to blood donations by humans. The duration of the procedures is a single donation but this may be repeated as permitted in the licence

What are the expected impacts and/or adverse effects for the animals during your project?

Blood removal may cause bruising/haemorrhage/haematoma at the collection site. From many years' experience the likely incidence is very low.

Less than 1% incidence of systemic reactions, e.g. anaphylactic shock, is expected or anaemia. During the previous 5 years of the licence no systemic reactions have been observed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Animals for blood donations only would be expected to have 100% mild severity.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Normal animal blood products can only be obtained from the species corresponding to individual clients' requirements.

Whole animal blood can be used to test mechanical devices such as mechanical hearts as it provides a more real to life comparison than with artificial blood. Blood cells for extraction of DNA to create naive recombinant antibody libraries require animal source for initial material.

Which non-animal alternatives did you consider for use in this project?

Customers requirements indicate animal sources of blood products.

Synthetic blood or blood products have been considered for some of the applications but they often do not mimic the characteristics of natural blood and it's products e.g. plasma and serum.

Why were they not suitable?

Synthetic blood or blood products have been considered for some of the applications but they often do not mimic the characteristics of natural blood and it's products e.g. plasma and serum.

No substitute for animal blood cells to extract DNA from to create recombinant antibody libraries. Once library is created, the requirement for animal products is removed leading to a long-term replacement for animal-based antibody production.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 sheep and goats have been estimated based on usage in previous years. About 250 sheep and 5 goats.

Alpaca normal blood products is a new product and is not expected to have a big demand initially. It is primarily for creation of naive antibody libraries, so single blood donation from each animal would be sufficient for each new library.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Normal animal blood products can only be obtained from the species corresponding to individual clients' requirements.

The required volumes of the blood and blood products, and hence the animal numbers required, depend greatly on their application. The animal numbers required will depend on the reviewed demand of the client.

Animals are sufficiently rested between donation events to allow a single animal to complete many donations throughout the life of this license, thus reducing the number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Larger sheep are selected for production and are better suited to our environment. Larger animals are able to donate larger blood volumes thus reducing their number of uses, and reducing the number of animals required.

For blood cells for recombinant antibody production, smaller volumes of blood are required to extract the cells, thus the number of animals required is reduced.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Large animals provide the most effective and economical source of blood products. They may be bled regularly with no ill effects. Large animals provide larger volume of antiserum at each blood donation to minimise the number of animals required to meet the necessary requirements.

Minimal rest period ensure the animals recover fully before another donation is collected. Having larger animals allows less frequent collections.

Total traceability and quality of our product is essential to the customer for their required use for the benefit of human health. The re-use of an animal which is continually under veterinary supervision is essential and this historical health screening ensures a product of the highest standard.

The use of animals such as sheep, goats and alpacas permits the husbandry of animals under high standards of farming conditions; with grazing in fields in the summer and indoor housing with feed supplements in winter. Their useful life is generally longer than commercially farmed animals. All animals are under the day-to-day care of an animal welfare officer and are visited frequently by NVS.

Why can’t you use animals that are less sentient?

Less sentient animals are unable to produce large volumes of blood donations to fulfill the requirements.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Use of animal conveyor system for handling, turning and weighing of animals: used for routine husbandry (e.g. dagging, foot trimming) and blood sampling. Safe efficient and calm system for both animals and handlers. Use of the same systems for routine husbandry and procedures allows animals to acclimatise over extended period of time before procedures begin.

Advances in animal welfare, care, husbandry, feeding, accommodation, and routine prophylactic treatment such as worming and vaccination ensure the animals are kept in a healthy condition for the prodedure.

Extensive daily monitoring of animals undertaking procedures. The animals will be restrained for bleeding which is planned and set up ready in advance which reduces the procedure time allowing the animal to return to its flock/herd quickly keeping the associated stress to a minimum.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Continuous improvements for procedures and training for operators undertaking procedures to maintain a high level of performance and minimise effects.

The Home Office Supplementary Guidance (HOSG) on projects for the removal of blood in accordance with the BVA/FRAME/RSPCA/UFAW Joint Working Group recommendations (2000).
NC3Rs: Experimental Design Assistant

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Subscription to the NC3Rs e-newsletter to keep up to date with the latest 3Rs developments. These monthly updates focus on funding opportunities, 3Rs events and publications.

Regular local and corporate animal welfare committees discuss 3Rs routinely with specific projects across the global organisation designed to implementing the 3Rs.

This establishment has the facility to browse a large and diverse range of publications via their parent company e.g. Journal of Visualised Experiments, Journal of Immunological methods.

122. The tumour microenvironment in cancer growth, metastasis and response to therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Tumour microenvironment, Therapy, Biomarkers, mRNA splicing

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study how cancerous cells communicate with normal cells inside tumours and how this causes cancerous tumours to grow and spread around the body.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer remains one of the leading causes of death world-wide, with one in three people dying of cancer in the UK each year. Although treatments have improved for some cancers, many still lack effective treatments and more research is needed before further improvements in survival will be seen.

Cancers that form solid tumours are the most common type. The spread of cancerous cells from these tumours around the body in advanced cancer, known as metastasis, is particularly hard to treat and is the predominant cause of death in cancer patients. The growth and spread of cancerous cells are dependent on a network of supportive signals. These are generated by the cancerous cell's interactions with normal cells recruited into the tumour from the surrounding tissue and the circulation. This supportive network is called the tumour microenvironment.

This project will study how the tumour microenvironment contributes to the growth and spread of cancerous cells from solid tumours and use this knowledge to identify new ways of diagnosing and treating cancer.

What outputs do you think you will see at the end of this project?

The potential outputs from the research outlined in this project are firstly to gain new knowledge concerning the role of the tumour microenvironment (TME), the cellular environment that surrounds cancer cells and is vital for their growth, in cancer biology. In particular this project will increase our understanding of the role of differential expression of gene variants within the TME and their impact on fundamental cancer biology. This project will also use information derived from these studies to identify new molecular process within the TME. Secondly this project aims to exploit this knowledge to improve existing cancer therapy by increasing our understanding of how this impacts on the TME and how it contributes to their effectiveness. Thirdly we will use the information gained to identify new therapies for targeted patient groups.

In addition to increasing the knowledge base surrounding the TME in cancer biology, we would expect this project to develop intellectual property that could be protected and further developed to provide novel therapies and prognostic and predictive biomarkers for the treatment of cancer. Information and knowledge generated during this project will form outputs including peer reviewed primary research articles and reviews articles, and presentations at workshops and meetings nationally and internationally.

Who or what will benefit from these outputs, and how?

We would expect the scientific community to be the primary, most immediate beneficiary of outputs from this project. In the longer term, we would expect out outputs to support improvements in cancer treatment through improved prognostic and therapeutic options for cancer patients.

How will you look to maximise the outputs of this work?

Results will be presented at national/international conferences and workshops, published in relevant journals and posted on the university's website, so that this information is made available to other scientists and clinicians with an interest in tumour biology within academia, research institutes, the pharmaceutical industry, NGOs and the public at large. We will also contribute to review articles and outreach activities in conjunction with local and national charities to make our research findings accessible.

Species and numbers of animals expected to be used

- Mice: 3300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As cancer is a systemic disease that impacts on the whole body, we need a model system that resembles human disease as closely as possible that allows us to study the impact of cancer growth and spread through the whole organism. This project will use mice as they are the least sentient animal whose physiology most closely resembles human disease. Mice allow us to study cancer across multiple parts of the body during cancer growth, metastasis and response to therapy. In the majority of protocols, we will be using adult mice in our studies. Occasionally juvenile mice may be used where we wish to activate specific genetic markers that will label the tumour microenvironment to aid our studies in cancer when the mice become adults.

Typically, what will be done to an animal used in your project?

In the majority of protocols (approximately 80%) mice will have cancer cells injected under the skin to grow tumours. These typically grow to a sufficiently large size to study in the laboratory by 4 - 5 weeks. During this time, we may treat mice with drugs to understand how the microenvironment within the tumour controls growth. We may also give localised radiotherapy to replicate human cancer treatment. Less commonly we may implant pieces of human tumour under the skin through a small incision.

These take longer to grow, sometimes as long as six months. Tumours grown under the skin do not spread very often and do not always closely resemble human tumours that occur elsewhere in the body. Where we wish to study cancer spread or need the tumour to better represent human tumours, we may inject the cells into different sites in the body. For example, we may inject cells into muscle or we may inject cells into a vein so they spread to other parts of the body. Less occasionally, we may need to inject cells into internal organs, for example into the liver to replicate the spread of colon or breast cancer, to better reflect human disease. When we cannot see the tumours to measure growth, we will label the cancer cells with a gene from the firefly that causes them to emit light. Mice will be briefly anesthetised so we can measure the amount of light being made to monitor tumour growth. Alternatively, we may use ultrasound to detect and measure tumours.

What are the expected impacts and/or adverse effects for the animals during your project?

When tumours are grown under the skin, mice usually show normal behaviour providing the tumour is less than 1 cm in diameter and it is positioned so it does not interfere with mobility, e.g., on the back. Occasionally the skin over the tumour may redden and scab, which can irritate the mouse. If this occurs, it usually happens near the end of the tumour growth period and mice are killed if this does not heal within 48 hours
Mice that have cancer cells implanted through a small incision in the skin or into an organ in their body usually recover quickly from the procedure, are generally able to move and feed within an hour and heal completely within a week to two weeks. To aid recovery mice are given pain relief and antibiotics. Mice are closely monitored and killed if recovery is not proceeding normally.

Mice often experience weight loss when tumours are growing in internal organs and this is monitored closely. Behaviour may also change and mice can become more withdrawn. These adverse effects are minimised through experimental design, particularly the use of methods for imaging and detecting tumour growth within the mice allowing the protocol to be ended before these impact noticeably on animal health and behaviour.

In the majority of cases, the drugs administered will not have a noticeable impact on animal health or behaviour. Some chemotherapeutic agents and radiotherapy can cause weight loss, as they do in human cancer treatments. These effects are closely monitored and doses that may give rise to noticeable weight loss or changes in behaviour are avoided unless there is a high likelihood these will have a significant impact on tumour growth. In most cases, treatment starts when tumours are detectable, typically within one to three weeks after the protocol starts and continue to the end of the protocol.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For mice with tumours growing under the skin, the majority (approximately 80%) will experience mild levels of discomfort or harm and the remaining mice no more than moderate levels of discomfort and lasting harm. Mice experiencing moderate levels of severity may increase when mice are treated with anti-cancer agents, particularly chemo and radiotherapy to approximately 50 - 60%.

A greater proportion of mice on protocols that include surgery or that lead to the development of metastasis will experience moderate levels of severity, and this could be as high as 80% of animals.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is a disease of the whole body and is regulated by complex interactions between the cancer cells and normal tissue cells recruited into the tumour, called the tumour microenvironment, that aid its growth and subsequent spread to other organs in the body. These interactions also control response to therapy.

This project aims to understand how these interactions control cancer growth, spread and response to therapy and then use this information to improve the use of existing therapies and to develop new ones. In order to achieve this, we need to use a model system that reflects human disease and response to therapy as closely as possible. Mice represent the

closest and least sentient animals that can accurately mimic the systemic nature of cancer and the tumour microenvironment so the aims of this project can be met.

Which non-animal alternatives did you consider for use in this project?

We have considered the use of isolated human cancer cells, the use of human cancer cells and normal tissue cells in co-cultures, including in three-dimensional culture, and cultured human tumour tissue as alternatives.

Why were they not suitable?

Although models of cancer using cells and tissues grown in lab culture are certainly useful, and we do use these extensively in the lab, they can only model limited aspects of cancer growth, spread and response to therapy. None are able to reflect the systemic, or whole-body nature of cancer, and lack perfusion with blood vessels and drainage by the lymphatic system that are important considerations if an accurate model of cancer is needed. These models also lack the complexity and variability seen within cancers growing in living mammals that have a direct impact on cancer biology and response to therapy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of mice have been estimated based on my previous experience as a Personal Project License (PPL) holder. This Project will support a similar number of investigators to my previous PPLs (approximately seven) conducting a commensurate number of protocols over five years (average three per year). Most investigators conduct pilot experiments using a small number of mice before moving on to protocols designed to test hypotheses (e.g., comparing different cancer cell’s ability to metastasise or response to therapy) using approximately 50 mice on average across three protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to reduce the number of mice used in this Project we have consulted the NC3Rs Experimental Design Assistant where applicable. We have also incorporated additional imaging methodologies like ultrasound that will allow us to monitor tumour growth within mice over time, reducing the number of mice needed for some experiments, in addition to using non-invasive imaging like luciferase activity (light production by cancer cells). Where transgenic mice have activatable genes within them to study cancer biology, these also activate expression of a fluorescent protein at the same time, allowing us to see our experiment is working in live mice, reducing the number of mice needed for optimisation of gene activation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will optimise the number of mice used by carefully controlling breeding of mice on this Project. Mice will be genotyped immediately after weaning and those not needed for protocols or further breeding will be killed. We also combine more than one female with each male reducing the number of mice used for breeding. Where possible, we will purchase mice from commercial breeders rather than breeding in house.

As I have held more than one PPL, I can draw on previous experience in planning experiments and protocols. This means we can accurately estimate the numbers of mice needed and in some cases, we do not need to conduct pilot experiments. We also use mathematical modelling to more accurately estimate group sizes. This can reduce the numbers of mice in one arm of a study when making comparisons between treatments.

Where we cannot draw on previous experience or modelling with confidence, we conduct pilot experiments with small numbers of animals. This includes optimising the doses, timing of new treatments or new methods of implanting cancer cells to study tumour growth or metastasis. These allow us to estimate the minimum number of animals for use in protocols to test our hypotheses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice and several different models of cancer growth and metastasis during this project. The reason for using multiple models and methods is to ensure we use the most refined approach that will lead to the least pain distress and lasting harm in order to meet our objectives. Cancer is a disease that proceeds through several stages before it causes death. By using different models and methods we can study these separately. For example, if we are most interested in growth at the primary site and early stages of cancer, tumours can often be grown under the skin to replicate this, where tumour growth causes minimal pain and distress and frequently do not spread to other sites. If this method will not allow us to meet our objectives, tumours may be implanted into more physiological sites, for example the muscle for sarcomas. In these models we minimise pain and distress by increasing monitoring of mice and ending protocols when tumours have smaller volumes than with tumours grown under the skin.

Where tumours need to be grown in physiological sites in internal organs, we will avoid making a surgical incision where possible by injecting through the skin into the organ directly, guided by ultrasound imaging if needed. On advice from the Vet, antibiotics and analgesia will be used peri and post operatively and mice will be carefully monitored. Growth of tumours in internal organs will be followed using non-invasive imaging techniques like bioluminescence, fluorescence and ultrasound. This allows us to stop the

protocol when the tumours are large enough to analyse, but not so big they lead to more than moderate levels of pain, distress and lasting harm.

We can often study growth of tumour cells at the most common sites of metastatic spread without having to grow a primary tumour and then wait for cells to spread elsewhere. For example, sarcoma normally spreads to the lung and by injecting cells into the tail vein we can get cells into the lung without needing to establish a primary tumour. For colorectal and breast cancer metastasis we can use ultrasound to inject cells into the liver, the common site of spread. As before we can monitor growth using non-invasive imaging techniques like bioluminescence, fluorescence and ultrasound.

Less frequently we may need to study how cells spread from the primary tumour to the secondary sites. In this case we can sometimes establish the first tumour as described earlier and use non- invasive imaging techniques like bioluminescence, fluorescence and ultrasound to detect spread to the secondary site. In some instances, the growth of cells at the secondary site can be very slow. In this case we may need to remove the initial tumour to allow the metastases to get big enough to study. To ensure mice experience no more than moderate degrees of suffering, pain and lasting harm, this method is limited to tumour grown at sites where they can be removed easily, or treated with localised radiotherapy to slow their growth, like under the skin. Again, if these are removed surgically, antibiotics and analgesia will be used peri and postoperatively and mice will be carefully monitored.

By using these approaches, we can generate tumours that are large enough to study and meet our objectives.

Why can’t you use animals that are less sentient?

Although less sentient animal models can be used to understand some fundamental aspects of cancer, for example zebra fish, the microenvironment within these models lacks the complexity seen in human disease in terms of stromal, or normal recruited cells. The least sentient animal suitable for our studies is the mouse. As a mammalian model, mouse tumour biology is a close approximation of human disease and the pattern of spread of cancer within mice often matches human cancer. As the cancers we are interested in are adult diseases we will also study them in adult mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In addition to increasing monitoring, we frequently use pre and post-operative analgesia and antibiotics to ensure recovery from surgery is rapid and as pain free as possible. We also refine surgical techniques through training and practice, reducing the incision sizes and sometimes remove the need for surgery all together where injections guided by ultrasound can be used. We also carefully consider what is needed to achieve our objectives so that welfare costs to the animals are minimised e.g., tumours are not grown longer or larger than is absolutely necessary, imaging methods are developed for models of metastasis to identify end points.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to the guidance published by the NC3Rs we also consult literature published by the Laboratory Animal Science Association (LASA), the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and subject specific guidance e.g. Refining

procedures for the administration of substances, Morton DB et al, 2001, Laboratory Animals "Guidelines for the welfare and use of animals in cancer research, Workman P et al, 2010, Br J Cancer and more recent publications including "Endpoint Matrix: a Conceptual Tool to Promote Consideration of the Multiple Dimensions of Humane Endpoints, Ashall V and Millar K, ALTEX. 2014" that encourage planning for unexpected harms during procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am registered with the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), am in contact with the NC3Rs regional programme manager based at our university. I also attend conferences and workshops organised by the NC3Rs and I encourage Personal Individual Licensees working on my Project Licenses to do the same. This enables us to keep up to date with the majority of advancements related to our research. We also regularly consult the published literature and attend workshops and conferences related to our specific field of research where information regarding new methods and procedures that lead to improvements in the 3Rs can be exchanged. If training is needed this is obtained through the staff within our animal unit or colleagues within our field of study. We regularly implement 3Rs improvements where they will not adversely affect our ability to achieve our objectives. This has included changes in the way we handle mice e.g., cupping or using tubes rather than their tails to pick them up, not using needles repeatedly and avoiding surgery to implant cells through improved methods or ultrasound to guide injections.

123. Studying glial cell regulation of central nervous system formation, function and dysfunction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

neuron, oligodendrocyte, zebrafish

Animal types	Life stages
Zebra fish (Danio rerio)	pregnant, adult, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this project is to use zebrafish as a model organism to better understand the mechanisms by which glial cells (support cells for nerve cells) regulate how the central nervous system is formed, how it functions, and how it can change to adapt or repair after damage. This work will help understand diseases of the brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We only have a limited understanding of the complex mechanisms that underlie the formation and function of our nervous system. This limits our ability to prevent or manage the effects of developmental, cognitive and degenerative disorders, which represent major societal burdens. Part of the reason for our limited understanding of the nervous system

lies in the vast complexity of the brain. The nervous system consists of an incredibly complex network of nerve cells (neurons), which are connected in a specific manner to form functional circuits. The formation and function of these circuits is orchestrated by intricate interactions between all cells that constitute the brain. About half of our brain cells are neurons, the electrically active and primary computing units that drive communication across our nervous system. The other half of our brain cells are support cells for neurons for which we use the umbrella term "glia" cells. There are different types of glial cells with different functions, from providing nutrition to neurons, allowing our neurons to talk to our immune system and our blood vessels. We are particularly interested in studying the functions of a glial cell type called oligodendrocytes. Oligodendrocytes are traditionally known to be the myelin producing cells of the central nervous system in all vertebrates. Myelin serves as an insulating coating around neuronal connections to enable proper signal transmission between neurons - just like the electrical insulation of a cable. Humans that don't form proper myelin or where their myelin is lost or damaged, as it is the case in neurodevelopmental and neuroinflammatory/degenerative disorders, suffer from severe neurological dysfunction. It therefore important to understand how oligodendrocytes make their myelin to help device strategies to improve these processes when they are faulty. In addition, there are large populations of tissue resident oligodendrocytes within the brain lifelong which do not form any myelin. The role of this cell population for brain function is almost entirely unclear, but recent evidence suggests that these cells have important roles for nervous system formation over and above their traditional role of making myelin. We use the relatively simple zebrafish as model system to discover the diversity of oligodendrocyte functions in shaping how the brain is formed, how it functions, how it is disrupted in disease, and how we might develop strategies to overcome dysfunction of the nervous system. The zebrafish is a vertebrate with many similarities to humans, including the presence of neurons and oligodendrocytes. Zebrafish also have an array of experimental advantages for studying nervous system formation and function in an intact living animal without the need of surgical intervention, allowing us to study the roles of neurons and oligodendrocytes and their various interactions with an accuracy difficult to obtain in other animals.

What outputs do you think you will see at the end of this project?

The work of this project will teach us how nerve cells (neurons) and nerve supporting cells (glial cells) of the brain interact to ensure normal nervous system formation, health, and function. We will also learn how neurons and glial cells respond to disruption to the nervous system, and how dysfunctional glial cells affect assembly and function of the nervous system when we create animals that model specific aspects of human diseases. Our ability to study zebrafish that model human disease also allows us to carry out discovery projects that aim to identify new targets as well as strategies to treat disease, be they manipulations to genes or drugs. We will publish our findings that describe our new insights in scientific journals, all of which will be open access to the public. In addition, we have generated and will continue to generate genetically altered zebrafish to share with researchers around the world. We will prepare press releases and social media-based outputs to convey the findings of our work to the public in a digestible manner. Our long-term goal is to help find treatments for disorders of the human nervous system, including neurodevelopmental disorders, disorders affecting cognition and mental health, and neurodegenerative diseases.

Who or what will benefit from these outputs, and how?

Numerous groups will benefit from the outputs of our studies. The scientific community directly involved in studying mechanisms of brain development and disease will benefit from new knowledge that can be integrated into our growing understanding of the nervous

system. Young scientists at early career stages will benefit from the training that they will receive by doing cutting-edge research to foster the next generation of excellent scientist in biomedicine in the UK. Pharmaceutical companies may benefit from our research by identification of new targets in our research that are of relevance to human disease. Therefore, we hope that patient groups, families, carers, and the wider community will ultimately benefit from our work, through the development of strategies to cure nervous system dysfunction. During the course of the project the general public will benefit from knowing that progress is being made in tackling major areas of unmet need in human health.

How will you look to maximise the outputs of this work?

We maximise the output of our work by collaborating widely with groups who have diverse expertise in technology or the use of different model systems. We collaborate with academic research groups that have complementary expertise (e.g. geneticists, systems neuroscientists) to help increase the impact of our work. We make our findings publicly available through open access publications, and by deposition of our work on pre-print servers prior to publication. In addition, we present our work widely at local, national, and international meetings, and through various media outlets.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 46,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All experiments will be carried out with zebrafish as model organism. Developmental, neuropsychiatric, and degenerative diseases of the nervous system represent a major burden to society, and there are currently very few treatments for diseases of the brain because we still lack understanding of the fundamental principles that govern its formation and function. This is in part due to the complexity of the brain and in part due to the difficulty of observing key biological events in real time in most animal models. Zebrafish help overcome many of these challenges. Zebrafish are vertebrates that exhibit remarkable conservation in terms of their molecular and cellular makeup with humans meaning that they often have shared mechanisms related to diseases and respond to gene and drug-based manipulations in similar ways. Importantly, the function of oligodendrocytes in zebrafish is highly conserved when compared with their roles in higher mammals, which allows us to use this model to understand oligodendroglial regulation of CNS function and dysfunction.

We primarily study young developing zebrafish in the laboratory because they develop quickly and are relatively simple, yet building a functional nervous system within a week when they also have oligodendrocytes as they communicate with surrounding neurons. At the same time, young zebrafish are very small in size (only about 0.5cm long) and thus optically translucent so that one can send light easily throughout the animal without need to any surgical intervention. These features, together with our ability to create genetically altered animals, and to treat young zebrafish with drug like compounds, means that we

can directly see into the brain and observe oligodendrocyte development, brain function and even brain pathology as it occurs over time. This then allows us to investigate how experimental manipulations, for example drug treatments, genetic and optical manipulations affect such processes in real time. Thus, the use of zebrafish allows us to gain insights into oligodendroglial control of the central nervous system that are difficult to achieve using other systems and to identify mechanisms underlying disease at an unprecedented scale.

Typically, what will be done to an animal used in your project?

The majority of animals that will be used in our project will be genetically altered zebrafish that have fluorescent proteins in cells or tissues of interest that allow us to track biological events in real time using a range of cutting-edge microscope and camera systems. Many of the animals that we use in our project will have further genetic alterations that change the function of specific genes of interest, e.g. a gene that can change the physiology of a cell using, for example, optogenetics to activate specific cells using light, or a gene associated with a human disease. In some cases, animals will be treated with small molecule compounds applied to the water to activate specific genes, to alter the state of a cell, or to induce the selective death of specific cells. Imaging experiments under different microscopes are always non-invasive and will typically be carried out under anaesthesia. Do do imaging, animals are typically embedded in a small drop of agarose to hold the animal in position while under anaesthesia.

In some cases when we need to directly assess CNS activity, animals may be embedded in agarose in the presence of neuromuscular junction blockers. In other cases when we need to assess behavioural outputs, imaging will be carried out without anaesthesia or neuromuscular junction blocker and the agarose is removed from the tail to allow free movement of the animal, or to monitor the animal as it is freely swimming.

What are the expected impacts and/or adverse effects for the animals during your project?

Due to the small size and rapid development of young zebrafish, we can carry out all our experiments in largely non-invasive manners, and typically with no signs of adverse effects on the animal. The vast majority of experiments will be non-invasive imaging carried out under anesthesia. These procedures may be repeated. However, we did in the past not observe accumulated impacts from repeated imaging as the animals recover fully from this non-invasive procedure.

In some cases, non-invasive imaging will have to be carried out on immobilised animals without anesthesia to assess nerve cell function and aspects of animal behaviour. These procedures may be slightly uncomfortable to the animal, similar to a human experiencing an MRI scan, but do not have any lasting adverse effects from on the animal. Sometimes, animals are restrained in the presence of neuromuscular junction blockers to prevent them from moving whilst under high-resolution microscopes. Again, these are acute non-invasive procedures where the animal will not experience any pain, but we cannot exclude a discomfort from being restrained.

We are, however, also interested in understanding diseases of the human nervous system, and so we have created models of certain aspects of human diseases using zebrafish. These include systems to ablate cells of the nervous system, or the alteration of gene function that changes function and the physiology of nervous system cells. In some cases, such animals can exhibit adverse effects including disrupted development, sensory

function or motor outputs. In the vast majority of cases, we study such animals at very early larval stages when they are about 0.5cm long and 0.1cm wide. Even though young zebrafish can exhibit sensory-motor functions which we study, they are at these stages not yet well developed so that it is not possible to measure adverse effects in great detail, which is different to rodents.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not study animals that exhibit severe adverse effects.

We occasionally study animals that exhibit moderate adverse effects, due to their genetic alterations, and because very few of our experimental protocols elicit adverse effects of their own. As our genetic alterations and experimental manipulations principally affect cells of the nervous system, moderate effects may manifest in altered neural circuit formation, such as impaired motor outputs leading to altered swimming behaviour. We would only study animals experiencing moderate adverse effects for short periods of time and only when scientifically necessary. We expect that less than 2% of all the animals that we will use during our project would experience this level of effect.

We also study animals that exhibit mild adverse effects. Such effects could be caused by the administration of anaesthesia, or mild stress due to being restrained during microscopy. We expect that up to 10% of the animals that we will use might experience this level.

Our experience to date indicates that the vast majority (>90%) of animals will not exhibit evidence of experiencing an adverse effect that is measurable. Most animals that we use are for breeding and maintenance reasons, and carry genetic alterations to visualise different cells of the nervous system with fluorescent markers. These alterations do typically not show any adverse effects due to their genetic alteration and are indistinguishable from wildtypes.

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 formation and function of neural circuits in the vertebrate central nervous system is incredibly complex. Brain development, function, and healthy maintenance involves intricate interactions between neurons, between neurons and glial cells and between these cells and our immune system and cardiovascular system. Many of these cellular interactions are not yet possible to study without animal models, because they are so hard to recreate in a dish. The consequences of damage or disruption to the nervous system

also triggers very complex cellular responses and interactions that cannot easily be reconstituted without animal models. Therefore, to be able to understand how neurons and glial cells interact to construct the nervous system, we need animal models to see how these cells are influenced by the immune system and vasculature in disease. However, in using zebrafish, we make use of arguably the simplest vertebrate model in which the complexity of the nervous system can be directly interrogated.

Which non-animal alternatives did you consider for use in this project?

Certain aspects of neuron and glial cell development, and even certain aspects of myelin formation by oligodendrocytes can be studied without using animals, by studying cells grown in the dish. I have spent my PhD research studying oligodendrocyte cell biology in culture dishes and have gained many insights from this work. Moreover, we continue to work closely with colleagues who use non-animal models. However, this current project aims to use the simple zebrafish model to begin to study the complexity of brain formation, function, and disease as it occurs in the natural setting. At present there are no other suitable non-animal alternatives to do so. We considered using cell culture techniques that now allow mini-brain-like "organoids" in the dish and are hopeful that in years to come technologies will become so refined that we they can be used to study neuron and glial cell interactions and neural circuit function. Such cell culture systems may even allow us to model certain aspects of disease in the dish, and we look forward to incorporating such models into our work.

Why were they not suitable?

Brain organoids are beginning to show great promise as an experimental tool for neuroscience, but have not yet been established to the point where they have all of the neurons and glial cell types that would be required to be able to probe the mechanisms of nervous system formation and function.
Furthermore, brain organoids have not yet been developed where they can function under physiological conditions with a vascular system, the full complement of immune cells, that are known to influence nervous system formation, plasticity, and repair.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

We have calculated the number of animals that we are likely to use based on our use of zebrafish as a model system over the past 5 years. One of the major advantages of using zebrafish as an animal model is that many distinct genetically altered lines of zebrafish can be maintained by single laboratories, and that the system can be used for large-scale discovery projects that are not feasible using mammalian models. Therefore, we plan to use a large number of animals through our project. Of the animals we project to use, that majority are for breeding and maintenance purposes. We have calculated the projected number of fish, based on our previous and projected use of approximately 250 tanks of zebrafish per week, in which we keep an average of 20 fish. We refresh stocks once per year, meaning that we will use 5 separate generations of each stock over the course of the

project, giving a total of 25,000 animals. We maintain >50 genetically distinct lines of fish, many used by several users, and we constantly monitor our stocks to ensure that we are not maintaining lines that are not in use.

In very few cases, animals will need to be maintained for breeding purposes where they may exhibit moderate adverse effects, which will be kept under protocol 2. We anticipate this to affect less than 2% of all animals used for breeding under protocol 1 and therefore project a maximum number of 500 animals here.

The third protocol projects the use of up to 6,000 zebrafish for the generation of new genetically altered animals. This number largely reflects the new ability to target gene function at scale using new tools, including "CRISPR-cas9" gene editing. We can now assess the effect of changing gene function in animals very soon after injecting reagents that can edit the genome. In the past, if we were interested in a gene's function, we would have to edit the genome and grow animals up to sexual maturity, maintain them through subsequent generations, and test if they affected a biological process of interest. Now we can look for the effects of disrupting gene function within days of such "editing". Although the number of animals we are likely to use may increase, the length of time that animals need to be maintained will be greatly reduced, representing an experimental refinement. The ability to target gene function directly means also that we can quickly assess how individual genes affect many different biological functions. For example, we can see how editing different genes affects different cell types or disease states because we can directly edit genes in animals with fluorescent reporters in their neurons, glial cells, immune cells, cardiovascular system etc. Testing the effects of 30 genes over the course of this project in up to 5 assays using up to 20 animals per assay will require 3,000 animals. We will also generate stable mutant lines from genes that exhibit particularly important functions when assessed by acute gene editing, and expect to generate up to 10 such lines, with current estimations that we need to grow up 50 animals to successfully do so (500 in total). In addition, we are currently generating animals in which gene function is disrupted in a cell-type specific manner. At present we need to screen through many animals to find suitable ones for in depth study, thus meaning we require about 200 animals per new line we establish. We anticipate establishing another 10 such lines over the course of the project. meaning that we will need 2000 animals for generation of cell-type specific mutants. In addition, we will continue to establish further new transgenic reporter and effector lines for which we need up to 15 animals per line. Thus, we estimate that we may use a total of up to 6,000 animals for the generation of new genetically altered animals.

In addition to the animals that we will study directly after their gene editing, we predict using a further 15,000 animals in our experimental analyses. This is driven in large part due to the ability to carry out in vivo imaging using transgenic reporter and effector animals. This enables us to have all experimental procedures being non-invasive, meaning that we directly observe and measure the intact living animal. However, it also means that a new animal is required for each cell type to be measured and/or manipulated because each individual animal can only bear a very limited number of transgenic reporters of effector (usually no more than two). Although we anticipate that many of our studies will be carried out at embryonic stages (5 days after egg fertilisation and under) before zebrafish are considered sentient enough to require legislative protection, we have established assays that do require analysis at protected stages after 5 days post fertilisation. For example, these include transgenic models in which we can ablate oligodendrocytes, i.e. cause demyelination, which we study after 5 days of age because myelin only forms in significant amounts during these later developmental stages. Similarly, studying neural circuit maturation and function requires analysis over 5 dpf as these are the stages when

zebrafish begin to feed and swim freely. Analyses of circuit function, and in particular the analysis of animal behaviour can we highly variable, so that we may need up to 50 animals per condition to obtain scientifically robust measurements. Therefore, we predict using up to a further 15,000 for other experiments, based on our current use. We carry out very careful calculations to define how many animals are needed to find statistically meaningful effects in our experiments and will continue to do so for new studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The principal step that we take in our experimental design is to determine what questions we can address using zebrafish at unprotected stages. The use of zebrafish at unprotected stages greatly reduces the number of animals on experimental protocols, and we will continue to pursue this strategy. However, the modelling of disease and the analysis of some aspects of neural circuit maturation are not possible to carry out at unprotected stages, nor are breeding and maintenance protocols, nor the generation of new stocks of genetically altered lines. However, we can assess the efficiency of transgenesis and gene editing at unprotected stages, which reduces the number of animals taken on to protocols. Another important way in which we can reduce animal number is through live imaging of individual animals over time. Through time-course or time-lapse imaging, we can gain a wealth of information about the dynamic nature of biological events from single animals that would otherwise require multiple animals being assessed at many different time-points.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to work to optimise the efficiency of transgenesis and gene editing, particularly cell- type specific gene editing, which we hope will reduce the number of animals that we use in our work. We will also work closely with our aquarium staff who are implementing trials that aim to adapt husbandry procedures to ensure more reliable sex ratios in our breeding stocks. Skewed sex ratios are prevalent in zebrafish stocks and definitive protocols to balance male and female generation have not yet been established. This would help reduce the number of animals that we need to use to generate sufficient breeding stocks for our experiments. In many cases, we will also be able to carry out pilot experiments on zebrafish that can be shared by other users who have the authority to do so.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use zebrafish to study neuron-glial interactions during nervous system formation, function, disruption and repair. We use zebrafish due to the ease with which one can

generate and maintain genetically altered animals with fluorescent reporters that allow direct visualisation of molecules and cells of the nervous system in embryonic and larval stages without the need of invasive procedures, and typically without any evidence of adverse effects. We also use zebrafish because of the ease of gene editing and manipulation of cell function. Again, the ability to carry out transgenic manipulations and gene editing on newly fertilised eggs means that their efficiency and any potential adverse effects can be observed prior to their development to ages that are protected. This is an important refinement that reduces animal numbers used and helps reduce any potential suffering the animal might experience, were it not possible to do so.

Our principal experimental methods involve live imaging zebrafish, and we can do so at different scales. We can carry out high-resolution screens of many animals to quickly assess how gene or compound function affect biological processes of interest, and in a manner that shows no signs of causing distress to the animal. In contrast we can also carry out extensive in-depth imaging over time of individual animals, either of the structure or function of their nervous system in a healthy or disease context. Time-lapse, time-course, of individual animals is a refinement, because we can gather enormous amounts of information from single animals. Such analyses provide insight into dynamic processes impossible to gain in other systems that would require the use of multiple animals if using other models.

We continue to pursue any innovations in husbandry practices, and when trialled and deemed successful, will be applied to our protected stocks, and may significantly reduce numbers of animals used for breeding and maintenance throughout the project.

Why can’t you use animals that are less sentient?

Oligodendrocytes are a vertebrate specific elaboration. Therefore, zebrafish are the simplest standard model in which they can be studied. Zebrafish also have an early onset of oligodendrocyte development and neural circuit formation and so are arguably less sentient than mammals by the time they have developed similar properties. We make every effort to study animals at the earliest stages at which we can address the questions that underpin the aim of our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Very few of our experimental protocols cause significant harm to animals and are already well refined. The main source of potential adverse effects to animals comes from the generation of new genetically altered animals where all possible effects on the animal are not possible to predict. However, as noted throughout, we can assess how new genetic alterations affect animals at unprotected stages and we carefully monitor animals following the introduction of new genetic alterations. We have also implemented a cutting-edge stock management database that will allow us to better track and monitor survival rates and any effects seen across all of our stocks.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines issued by the National Centre for the 3Rs, and will follow the recently published guidelines put together by a group of zebrafish researchers in collaboration with animal welfare experts at the Federation of European Laboratory Animal Science Associations (FELASA).

In addition, we continue to refine practice across all experimental approaches as innovations and advances are published in the literature.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My group and I follow NC3Rs on social media, and stay informed about relevant innovations through our proactive bioresearch and veterinary services at the establishment. We will implement appropriate advances through discussions with our local vets and named animal care and welfare officer. In addition, we plan to propose research proposals to NC3R to actively contribute to the goals of NC3R in biomedical research.

124. In Vivo Imaging and Studies in Cancer Disease 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
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Imaging, Diagnosis, Therapy

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate
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's the aim ofthis project?

This project aims to develop in vivo imaging methods and agents to improve diagnosis and treatment of cancer. A variety of novel imaging technologies, combinations thereof and therapies will be assessed for utility in cancer management.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
In the UK, more than 367, 000 patients are diagnosed with cancer each year. Further

improvement in the management of cancer patients requires: early detection of small tumours and metastases (cancer spread), informed treatment selection, outcome prediction, and monitoring of treatmentoutcome.

Imaging techniques can non-invasively detect disease and measure response to treatment.

The project will allow us to develop novel imaging techniques and devices that will help to better diagnose, understand and quantify the severity of cancer development and also improve current interventional procedures for the treatment of cancer. As a consequence, we aim to more efficiently treat patients with cancer and help to assess how patients respond to treatment (thus avoiding debilitating and expensive treatments in patients who will not benefit). This knowledge is important and valuable as it will help us in identifying patients that are at high risk of cancer spread and provide the appropriate treatment to patients based on more quantitative and objective measures.

Ultimately this work aims to improve cancer treatment by early detection and improve monitoring of cancer spread over time.

What outputs do you think you will see at the end of this project?

This programme will develop the requisite: new imaging contrast agents and improved chemistry for their synthesis, and the imaging instrumentation and methodology, for application in cancer.

In the context of this licence, a contrast agent is any administered substance which permits differentiation of structures or fluids within the body, (generally detectable by imaging devices), for the purposes of identification, diagnosis and monitoring of disease evolution or response to treatment.

We will develop contrast agents for molecular imaging of processes that hitherto could not be imaged, develop improved agents so that known processes can be imaged better and develop new chemical methods for synthesis of the agents so that they can be produce more simply and cheaply. These new technologies will undergo a “bench to bedside” translational process in which small animal imaging is a key component to validate the targeting principle employed, predict efficacy, and provide information required by the regulatory agencies to allow assessment of the methods in humans. The new agents must be validated, demonstrating that the images truly reflect the process in question and its response to treatment.

We will develop new imaging instrumentation to combine modalities synergistically, potentially compensating for inadequacies of individual modalities and exploiting their complimentary advantages.

Who or what will benefit from these outputs, and how? Short-term:
Better animal model development: The use of pre-clinical imaging allows us to monitor and evaluate tumour model development particularly in deep seated organs such as ovaries, lungs or brain to help us understand tumour evolution prior to appearance of significant clinical symptoms. Thus, we may then study our novel contrast agents at earlier points in the disease evolution, and thereby achieve our scientific aims at earlier humane endpoints. In addition, better understanding of molecular processes during tumorigenesis (e.g. blood vessel development, regions of dead vs living tissue within tumour masses, up-regulation or down-regulation of receptor expression during disease progression and it's

consequences for targeted treatments, tumour cell spread, impact of treatments/therapy) and dissemination of this data, will increase our basic knowledge as a scientific community.

Less animals used: Repeated imaging provides us with the capability to image an individual animal much like a human during its disease progression and treatment. By using this technology or indeed using multiple imaging technologies, we can obtain far more data per animal than would otherwise be possible using traditional scientific experimental designs.

Evaluation for progression towards human clinical trials: The immediate benefits of research under this licence would be to allow us to make an informed decision whether to test a new contrast agent or therapy in humans, or return to cell studies or chemical laboratory for further modification. The same is true for the evaluation/development of novel instrumentation, computational methods and combinational imaging modalities to asses their suitability for progression to human clinical trials/studies.

Medium/Longer term:

Chemistry & manufacturing: By developing and evaluating better contrast agents, the quality of imaging data will be improved (e.g. improving affinity for target) and give better detectability of smaller amounts of disease. For example, in the field of radiochemistry (for diagnosis and/or treatment), by making radiosynthesis and purification of agents simpler and more robust will lead to shorter production times. In turn, this will result in wider availability to more hospitals without the need for costly cyclotron, radiochemistry equipment and radiochemical expertise in situ. Thus with more widely available contrast agents, more patients will benefit from the technology available.

Therapeutic delivery and monitoring: In addition, imaging also has the potential to non-invasively evaluate therapeutic efficacy in patients, providing rapid feedback on therapeutic or interventional effectiveness.

Novel instrumentation, computational methods and the combination of imaging modalities will benefit both patients and health services through improved diagnosis and clinical decision making.This has already been demonstrated through development of PET/CT and SPECT/CT which are now in routine practice. Clinical MR/PET and MR/SPECT are on the horizon and will extend application of these principles while reducing radiation doses to patients through avoidance of CT scanning. The immediate benefit that the experiments will provide is the basis for authorised translation of these techniques into clinical use.

Whether directly by the development of new imaging technologies/contrast agents, or indirectly by use of imaging as a tool in basic biomedical research, better quality and wider availability and applications of imaging technologies will lead to better clinical decision making and better quality of life for patients, reduced drug development costs, and reduced costs for health services. The beneficiaries will be patients, health services and pharmaceutical companies.

How will you look to maximise the outputs of this work?
We will closely collaborate with academic, clinical and industrial partners to ensure that the developed imaging probes can be tested in human trials and ultimately be commercialised to ensure widespread clinical use of the developed probes for patient benefit.

Similarly, we will closely collaborate with industrial partners to develop prototype software and hardware that can be subsequently shared with other academic centres for wide spread clinical testing.

We will also disseminate the results of this work at conferences and workshops and publish in peer reviewed national and internal scientific journals. In addition, we will organise internal workshops where we will share our results, unsuccessful approaches and provide hands on training for internal and external academics interested in this work. Other means of dissemination will include public engagement events such as talks at the Pint of Science initiative or active participation in the summer festival of the Royal Society.

Species and numbers of animals expected to be used

- Mice: 8000
- Rats: 1750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are the mammalian species of lowest neurological sensitivity that provide the necessary size compatible with the scale of resolution or movement associated with the techniques being studied. Resolution of the whole body imaging techniques is of the order of 0.5 – 1mm. Distribution within smaller animals will be beyond these limits.

We will generally use young adults for our studies. Older mice may be used, particularly if these are genetically modified strains which develop cancer spontaneously as the animal ages. Animals may be allowed to age within limits, if we are studying the impact of therapy/surgery on tumour re-occurrence.

Typically, what will be done to an animal used in your project?
Generally, over a period of 2 - 3months:

Induction of tumour or implantation of cells/tissues in genetically altered animals/wildtype animals (may take 2 - 6 weeks for establishment) or use of a transgenic spontaneous cancer model (may take weeks or up to 6 months for this model to be established).

Injection of contrast agent generally under anaesthetic (typically by intra-venous injection; anaesthesia may not always necessary); in some cases, an additional molecular or cellular agent may be administered, e.g. an inhibitor of the specific process, to modify the biodistribution of the contrast agent. This could be administered via a variety of routes, ranging from intra-peritoneal to possibly through food/water.
Imaging by PET/CT, SPECT/CT, MRI, Optical or other imaging modality, under general anaesthetic.

Less frequently, mice may be housed singly in cages with a grid floor post- step 2 or 3, to collect radioactive faeces/urine for no longer than 3 consecutive days to obtain more complete information regarding the fate/stability of the radioactive contrast agent (e.g. a long-lived antibody).

In some cases both steps 2 and 3 may be repeated to obtain time dependent data or to image different stages of the induced/spontaneous disease or disease response to therapy. Therapy can be radio/chemo/biological therapy.

The final imaging session will be under terminal anaesthetic and will generally be followed by tissue explantation for further data extractions e.g. radioactivity measurement to provide additional quantitative biodistribution and dosimetry data and/or histology.

Rarely, animals may undergo surgical removal of the primary tumour, if the aim of the imaging experiment is to assess the efficacy of the surgery (without optical enhancement) and the re-occurrence of the tumour over time.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect to observe adverse events in the different tumour model induction, development and also during therapy.

Almost all tumour induction models (bar spontaneous tumour development) would be done under general anaesthesia. This would include, administration of cells via intravenous injection or administration post surgical exposure of target tissue/organ. However painkillers given before, during and after any surgery to reduce pain and discomfort.

A superficial tumour may not cause any adverse events but dependent on its growth, the skin surface could redden due to inflammation or become thin and breakdown.

Generally, we expect to see changes in animal well being as tumours progress or metastasise: possible weight loss, hunched posture, ruffled coat and subdued behaviour. Specific cancer models will be prone to particular known adverse effects, e.g. lung tumours would be expected to affect breathing, ovarian cancer is known to lead to visible distension of the abdomen, bone cancer may lead to less weight applied to affected limb or brain tumours may cause head tilting or seizures. All such adverse events will have humane endpoints to prevent animal experiencing clinical signs exceeding Moderate severity limits.

Administration/application of certain therapeutic strategies are known to lead to adverse events. Some chemotherapeutics and radiotherapy regimes are known to cause changes in well being as described above or development of diarrhoea as known to occur in human patients.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals under this licence are expected to experience a Moderate Severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We require the use of animals because:

Data generated from this body of work may be used to inform whether to go forward to human clinical applications. Regulatory agencies require animal data to demonstrate safety and efficacy before molecular imaging agents or therapeutics (that can be validated through imaging) can enter human trials.

To validate the mode of action new/improved molecular imaging agents, experiments are required that cannot be conducted in humans for ethical and scientific reasons.

Bio-distribution in whole organisms (i.e. tracking the injected agents route/ accumulation and excretion through the body), with intact biological barriers and excretion mechanisms, is key to clinical use.

Most aspects of cancer can only be studied in live animals because there are complex interactions between different body systems, which cannot be truly replicated in anything other than an intact animal.

Which non-animal alternatives did you consider for use in this project?

In some limited cases, absolute replacement using humans is a possibility, e.g. when contrast agents that are already used in humans are evaluated for clinical utility and uptake mechanisms. If this is feasible and allowed by the regulatory agencies we will do so.

Prior to all in vivo work, human and animal cell- and tissue-based methods will be used as relative replacements to answer as many research questions as possible and build solid hypothesis to be subsequently tested in vivo. This includes:

experiments in the laboratory, designed to determine target-binding efficiency, agent toxicity to cells, agent stability in cultured cells/tissues/serum;

Within our School we are working on developing the chick chorioallantoic membrane (CAM) as an alternative, high-throughput method for the development of novel cancer imaging agents. The CAM is a highly vascularised extra-embryonic membrane of the chick embryo. The CAM can be accessed easily with minimal invasion to the embryo, enabling the growth of cultured cancer cell and patient-derived xenografts, complete with a co-opted vascular system. This may serve as a replacement for some animal experiments in the future.

'Phantoms' are objects used as substitute or mimic for human tissues to ensure that systems and methods for imaging the human body are operating correctly. So 'Phantom' experiments will be to demonstrate function and capability of the new instruments prior to further testing in live animal settings.

Any new/improved molecular imaging agents or instruments, which are found by such experiments to be unlikely to succeed in animal or later human trials will be eliminated at this stage.

All alternatives are limited in the amount and quality of data they can provide.

Why were they not suitable?

Alternatives are not suitable because such non-living animal/human alternatives cannot replace the complexities of the interactions of these probes in whole body systems or with realistic models of cancer.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The preclinical teams within our School have worked for more than 12 years on similar projects using tumour models. Therefore, based on our extensive experience using such tumours, we estimate this number based on previous experience.

We also have new grants which have been award following peer review and we have factored in the types of experiments and animals proposed therein to account for future work over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As we have access to our archived imaging data from the past 12 years, it is possible to re- examination/re-evaluation this imaging data. By doing so, we may be able to avoid starting from scratch and running a pilot experiment, thereby reducing animal numbers.

Our researchers are able to access online tools such as the NC3Rs Experimental Design Assistant during experimental design but all experimental protocols are also assessed by a number of colleagues (group leaders, myself/imaging scientist, other collaborative scientists, NTCO, NACWO) from inception to final approval.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our animal unit has implemented a new Mouse Colony Management system which is leading to efficient breeding management of colonies through better and clearer data generation.

Imaging to determine tracer distribution rather than conventional ex vivo organ counting is a major contributor to reduction. It allows repeated time-dependent measurements on the same animal as animals are only killed at the last time-point; for example, if a study

involves six time-points, the animal numbers are reduced to one sixth. Since each animal serves as its own control, the data are statistically also more robust, which in turn leads to further reduction as smaller cohort sizes are required (because inter-animal variability no longer needs to be considered at the experimental design stage). Moreover, not only contrast agent distribution in vivo, but potential time-dependent and unexpected re-distribution can be detected through serial imaging. All these attributes contribute to a greatly improved benefit: cost ratio (benefit=data quality/quantity, cost=animal numbers/procedures).

Using imaging technologies and pilot studies allow us to use far few animals in determining the best experimental design to 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Species: Mice and rats are the species of lowest neurophysiological sensitivity that provide the necessary size compatible with the scale of resolution or movement associated with the techniques being studied. Resolution of the whole body imaging techniques is of the order of 0.5 - 1 mm. Distribution within smaller animals will be beyond these limits.

Pilot studies are small experimental groups which help us to decide quickly how best to design a statistically and scientifically valid experiment. Thereby helping develop better larger study design and reduce possible suffering. Generally, inhalation anaesthesia will be used to minimise any transient pain or distress and where possible, used for blood sampling, contrast injection, weighing and combined with imaging techniques where it is mainly used for restraint. In addition, there would be full and complete recovery between periods of anaesthesia and/or food withdrawal; rehydrating of animals during long imaging sessions; monitoring of respiration and/or cardiac function and maintaining body temperature during imaging. These steps/measures will optimise the animal's welfare whilst undergoing these procedures. By the very nature of the work involved in this project animals will develop cancer and may undergo therapy. Therefore, careful monitoring will occur throughout the life- span of an animal undergoing these procedures and appropriate interventions will be made to control, reduce or prevent adverse events.

Prior to development of 'orthotopic' tumour models (placing the tumour in the organ where it would naturally develop , e.g. lung cancer), we will assess the tumour growth in a sub-cutaneous (under the skin) model first. By doing so, we would also be able to ensure that the contrast agent being used can truly target the tumour tissue and is useful to study further. Sub-cutaneous tumour models are less likely to cause adverse events to the animal and allow easier visual monitoring of the tumour. Only agents which are likely to be successful will be tested further in tumour models where the tumour develops in it's preferred organ. However, generally adverse events are more likely to be seen in the animal, e.g. a lung tumour could eventually interfere with breathing and is more difficult to

monitor as it grows.

Pain relief will be administered as required, (always in association with surgical procedures) and under veterinary direction. Animals will be humanely killed at the end of the experiment or before then, if the humane endpoint is reached and tissues used for further examination.

Why can't you use animals that are less sentient?

Studies in less sentient animals cannot be used to replicate the complexity of tumour development, metastasis and therapy which could be applied directly to humans.

It is not possible to study tumour development or response to therapy in animals which have been terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

It is a part of PPL conditions and our ethical understanding that we let animals only develop tumours to a level of burden required to answer the specific research questions of each individual experiment, for example:

if initial in vivo experiments are performed to investigate the uptake of a new contrast agents in early disease, the answers can be obtained by the presence of relatively small tumours without the need of advance disease or clinical signs; the use of repeated imaging in disease progression and treatment experiments where we then can quantify tumour burden by imaging and obtain statistically better data (repeated imaging reduces suffering by reducing the required animal cohort sizes). In fact, imaging will help us in many cases to better assess the overall disease burden in our animals. Once an individual experimental goal is reached the animals will be culled regardless of whether the endpoint has been reached.

We always use objective animal monitoring to allow us to develop better practices regarding better identifying animal discomfort, pain or distress but which also allows us to bring an imaging session forward and cull the animals prior to it reaching a humane end-point.
We always induce tumour model establishement under general anaesthesia and administer analgesia prior to and post any surgical intervention under NVS guidance.

Inhalation anaesthesia will be used wherever possible to minimise transient pain and distress, e.g. during imaging. In addition, full recovery between periods of anaesthesia, rehydration during long imaging sessions, respiration/cardiac function monitoring, body temperature monitoring/maintenance will be conducive to animal wellbeing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow established published guidelines to ensure experiments are conducted in the most refined way. These includes:

The Responsibility in the use of animals in bioscience research produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

The Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

Accepted limits of volumes and frequencies when administrating compounds and anaesthesia (Appendix 1a in Action Plan section).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project? We will stay informed by updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) website and seminars on the 3Rs organised within and outside of our institution.

Additionally, we have direct support and contact with an NC3R's regional Programme Manager who supports the application of the 3Rs within our institution and is a member of the Policy and Outreach Group. This includes providing expert advice and coordinating the sharing of best practice.

I am the Chair of our Campus AWERB and therefore have access to the latest developments, advancements and information relevant to this project.

125. Understanding fundamental aspects of thymus development and function

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Thymus, T cells, Immunity, Stromal Cells

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to improve our understanding of the basic cellular and molecular mechanisms that control T cell development within the thymus.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 thymus is a unique organ situated in the upper chest that supports the development and export of mature T cells. T cells are a highly specialised type of immune cell that provide immune protection against infections such as bacteria and viruses, and additionally protect against cancer formation. The importance of the thymus to the generation of protective immunity is illustrated in individuals that lack normal thymus function and T cell development, with such individuals possessing a significantly enhanced risk of developing potentially life-threatening diseases. Despite the essential requirement for normal thymus function in generating effective T cell-driven immune protection, the basic mechanisms that control i) the development of the thymus and ii) how it is uniquely

able to support the development of functional T cells remains incomplete. In this programme of work, we aim to undertake fundamental, exploratory research that is targeted at increasing our basic understanding of the cells and molecules that play key roles in thymus development and function. This programme of basic exploratory research is anticipated to contribute to our knowledge base of how T cell development is regulated and may therefore ultimately provide a platform for the subsequent long-term development of therapeutic strategies targeted at manipulating the thymus and T cell immunity when this is dysfunctional.

What outputs do you think you will see at the end of this project?

Work performed under this licence will:

Generate basic science data that will enhance our fundamental understanding of the cellular and molecular mechanisms controlling thymus function and T cell development.

Provide a platform for the identification of new pathways and targets that may inform follow-on studies that are targeted towards the development of translational approaches to manipulate and enhance thymus function.

Generate scientific findings that will be communicated to the wider public and scientific community through presentation at scientific meetings, public engagement activities and publication in high impact peer-reviewed scientific articles.

Who or what will benefit from these outputs, and how?

The outputs from this will be of interest to a wide range of individuals and groups interested in understanding thymus function, and exploring the potential for manipulation of thymus tissues in order to enhance T cell development and output. It is anticipated that the immediate beneficiaries of this research will include basic research scientists within the field of biomedical research interested in T cell biology and the impact of perturbed T cell development on immune protection. At a local level, interactions with both clinical and non-clinical colleagues will ensure discussion and distribution of data and findings from the project. Wider academic beneficiaries will be engaged through sharing of information through attending and presenting data at national and international meetings. Results from the project will be published in free-to-access scientific publications.

Importantly, this basic science project sits at the discovery end of the translational pathway. However, it is essential that such exploratory projects are undertaken if we are to identify novel targets and thereby make new advances in the potential for manipulating the immune system for therapeutic benefit.
Therefore, although outputs from the project may be of interest to the clinical community in the short term, the likely potential for any outputs to directly inform future therapeutic approaches should be viewed in the long term. In addition to individual beneficiaries, the ultimate potential long-term goal to translate research findings into methods to manipulate immune function in humans may have socio- economic impacts due to the health and wellbeing implications of poor immune function.

How will you look to maximise the outputs of this work?

In order to maximise outputs from this work, we will continue to maintain existing and develop new collaborations with clinical and non-clinical colleagues at both national and international levels. Such collaborative interactions are anticipated to enhance sharing of

both knowledge and materials that will provide added value to both this programme of work and others. Outputs from this programme of work will be shared with the scientific community through communications of results and technical approaches at meetings and conferences e.g. by both oral and written presentation. Such dissemination of work will raise the profile of work and allow sharing of data outputs that will enhance knowledge exchange and support development of our projects via feedback from experts in the field. In addition, we will continue to publish our results in high-impact journals. Importantly, in line with the requirements of our funding bodies published results will be in open-access scientific journals in order to make our research as accessible as possible. We also aim to publish negative results where these will provide important data that may inform other studies or prevent replication of experiments elsewhere. In order to communicate outputs with wider audiences we will continue to use additional platforms, including social media e.g. Twitter to maximise exposure of results and publications. Where possible we will also engage with the wider public through public engagement events in order to communicate our work to wider audiences.

Species and numbers of animals expected to be used

- Mice: 18,400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The immune system is formed by a network of cells and inter-connected organs that are spread throughout the body. In this programme of work we aim to look at how the thymus supports development of T cells and how these are exported into the peripheral circulation in order to provide protective immunity. In particular, we aim to study thymus development and maintenance throughout the life- course and investigate the pathways that endow the thymus with its unique ability to support T cell development. In order to investigate these aims, we will use mice in this project. The mouse represents the lowest order of placental mammal that possesses an immune system of similar make-up and function that can be used to effectively model the human immune system, including the thymus. In addition, mice represent the primary species that possess a wide range of naturally occurring and induced genetic defects that allow us to study the role of specific cells and molecules in thymus biology. As this project seeks to explore how the thymus initially develops and is subsequently maintained we will use mice at multiple stages of the life-course including fetal, neonatal and adult.

Typically, what will be done to an animal used in your project?

This project licence will support the breeding and maintenance of mice that possess alterations in specified genes (herein termed genetically-altered). The use of genetically-altered mice will underpin experiments aimed at exploring the role of specific genes or cells of interest in thymus function. In order to do this, we will breed genetically-altered mice that have altered expression of defined genes. At various stages of life, mice will be humanely killed and cells/tissues will be isolated for direct post- mortem analysis without any additional procedures being performed on animals. Such experiments will allow us to experimentally examine whether thymus function and T cell development is perturbed

where expression of the gene of interest is altered.

Where the above experiments reveal a disruption in thymus development or function, we will perform further investigations to explore how the gene of interest is exerting an influence on thymus biology. These experiments will involve animals experiencing one of the following approaches:

Animals will receive an injection, typically this will be a single injection, although in some cases may involve up to 6 injections separated by at least 24 hours over a maximum period of 2 weeks. Mice will then be humanely killed at a later time point for analysis of tissues post-mortem. The majority of animals will undergo a peripheral injection (for example into the tail vein), but some will receive an injection directly into the thymus; this is a surgical approach that will be performed under general anaesthesia. Thymus injection will only ever be performed on a single occasion.

Animals will be transplanted with donor thymus tissue placed under the kidney capsule on one side of the animal. This involves a short surgical procedure and will be performed under general anaesthesia. Animals will be subsequently humanely killed at a later time point and tissues will be analyzed post mortem.

What are the expected impacts and/or adverse effects for the animals during your project?

Brief stress due to temporary handling of mice and discomfort due to needle insertion required for the delivery of cells OR substances (typically labelling agents or proteins/peptides) by injection into peripheral sites.

Recovery from general anaesthesia following EITHER a single injection into the thymus (intrathymic injection) or surgical transplantation of thymus tissue under the kidney capsule.

It is not anticipated that any genetic alterations in mice used in this project will have downstream adverse effects. In addition, in animals undergoing thymus injection or tissue transplantation we do not anticipate any adverse effects arising from the grafted tissue. Transplanted tissue will be genetically matched to the host to avoid rejection or additional complications of transplant. Animals are expected to make a full recovery following injection or transplantation of cells/tissues and are expected to exhibit normal behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The upper severity of an estimated 80% of animals will be mild. This encompasses the breeding and maintenance of genetically-altered animals, followed by post-mortem analysis of cells/tissues, and also animals undergoing a single mild intervention involving an injection of cells OR substances (typically labelling agents or proteins/peptides) into peripheral sites.

It is estimated that 20% of animals will experience an upper severity of moderate. This includes animals undergoing surgical approaches involving general anaesthesia, encompassing EITHER a single intrathymic injection OR transplantation of thymus tissue under the kidney capsule.

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 analysis of T cell development and thymus function relies on the coordinated activity of many cell types and molecular signals that require the use of whole animal models to investigate in detail. For example, the thymus does not contain haematopoietic (blood) stem cells that develop into T cells.
Rather, blood stem cells are located in the marrow of skeletal bones and migrate from these distant sites in the body through the bloodstream in order to colonise the thymus. Importantly, this is a continual process meaning that the study of T cell development relies on an interconnected system of skeletal and circulatory blood vessels with the thymus. Following development in the thymus, a critical step is the export of T cells into the peripheral circulation in order to provide effective immune responses.

Similar to thymus colonisation, in order to study this critical step it is necessary for the thymus to be fully connected to an intact circulatory system. At present no *in vitro* experimental systems exist that can effectively model this complex, interconnected network of the circulatory and skeletal systems with the thymus.

Which non-animal alternatives did you consider for use in this project?

We routinely use in vitro (in a dish) models in our research, including cell and thymic organ culture systems. These approaches include thymus organ culture systems. These models help to reduce the need to perform experiments in whole animals, and provide a key system where we are able to validate experiments in vitro e.g. screening and testing the ability of particular molecular pathways to influence thymus function before these are explored in animal models. Isolated thymus tissues will be placed in in vitro culture where they can be maintained for up to 10 days and are able to replicate a normal programme of thymus and T cell development, at least for a limited short term period of time. Using this system, it is possible to test the role of defined molecular pathways in thymus function by introducing agents that can either stimulate or inhibit pathways of interest. In addition, using in vitro ‘tissue engineering’ approaches in these thymus culture systems (similar to organoid models), we are able to isolate defined cellular components from the thymus and mix them back together where the cells are able to spontaneously reassemble back into a mini thymus in a dish. This model provides a useful in vitro replacement model where we are able to create a ‘designer’ thymus in a dish. Using such systems we can track the development and function of cells within the thymus.

However, current limitations to such in vitro models, such as lack of a connected blood supply and therefore absence of immature T cell recruitment from the bone marrow, currently place limitations on the use of such models as a complete replacement for whole animal models. A further current limitation to the use of non-animal models for thymus research remains the lack of information regarding stem cell populations that are capable of

generating functional thymus tissues. Although our previous work, and that of other research groups, have sought to identify stem cell populations that can form complete thymus tissues, as yet no specific markers have been identified that allow for the isolation and propagation of such cells in vitro, thereby precluding their use in alternatives to animal models.

Why were they not suitable?

The thymus contains a unique three-dimensional network of cells that support the development of T cells. At present, attempts to use cell culture models to recapitulate normal thymus structure and function, in particular use of 2D cell culture models has met with limited success. The lack of specific markers to isolate stem cells from thymus tissues in order to attempt generate thymus tissues has also hampered efforts to replace animal models. Recent efforts have also been made to utilise organoid systems to study aspects of thymus biology, although as yet the effectiveness of such systems remains limited and have failed to model normal thymus development and function. Critically, the need for a fully integrated immune system that communicates and directs the migration of immune cells through a system of blood vessels is essential for the study of the thymus and T cell development. As such alternative models to study thymus function currently remain limited. However, we will continue to study the scientific literature for any emerging models that may facilitate reduction and replacement of animal models, and will continue to utilise and refine our current use of non-animal models such as thymus organ culture systems 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 programme of work is a continuation of work performed under our previous project licence. We have therefore estimated our numbers based on our experience of animal husbandry and use of animals using identical protocols and procedures over the last 5 years of our previous project licence. Numbers of animals are therefore based on previous Home Office data returns for the use of animal numbers, in-house data from pilot studies and ongoing/published studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design phase we use power calculations (e.g. using G* Power software) to estimate the predicted group sizes necessary to determine statistically significant results. In order to reduce animal use, we routinely use robust approaches to ensure standardisation of experiments in order to make them directly comparable for example where an individual scientist repeats an experiment across different days, or where experiments are performed by more than one individual. We adopt standard experimental approaches within our research group to ensure reproducibility via the use of shared experimental techniques and regular lab meetings to ensure that all research lab

members are performing experiments in exactly the same way. We also perform regular calibration of our scientific instruments again in order to reduce the need to repeat any experiments due to variability in the acquisition and recording of results.

Wherever possible we use control animals from the same litter as experimental mice in order to both reduce unnecessary animal breeding and to reduce the potential for any observations occurring as a consequence of differences in genetic and/or age-related factors. Where undertaking a new series of experiments we will perform pilot experiments coupled with power calculations to estimate the number of animals that we need to breed, maintain and subsequently use in experiments. We will also make use of NC3Rs resources for 'Experimental Design and Reporting' (https://nc3rs.org.uk/experimental-design-and-reporting) to support our studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to optimise our use of animals, we will use a combination of approaches:

Efficient animal breeding and husbandry. Mouse colonies will be maintained at a size necessary to generate animals required for robust experimentation. Wherever possible, we will use litter-mates as control mice in order to optimise maximum use of animals generated under this licence.

Pilot studies. These will be undertaken whenever we commence a new series of experiments or use new reagents etc. Outputs from such experiments will allow us to accurately plan animal breeding and subsequent experiments for example through the use of power calculations.

Sharing of materials. We undertake regular team research meetings at the start of every week where we discuss experimental plans. Here we are able to maximise use of every animal by combining experiments and sharing tissues/cells taken from each animal.

Archiving materials. We regularly archive cells and tissues by freezing and storing materials. Such archived tissues provide a valuable resource for future experiments, for example gene expression and microscopy analysis of cells/tissues. Using such approaches we are able to maximise use of tissues and reduce the need to breed additional animals.

As described above, we will make use of NC3Rs Experimental Design and Reporting Resources to guide our studies, and will ensure experimental design and conduct is performed and reported in line with the ARRIVE 2.0 and PREPARE guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm

to the animals.

This programme of work will be underpinned by the breeding and maintenance of genetically-altered mice in order to explore the cells and molecules that control thymus development and function.

Injection of cell or substances

Where we have identified candidate pathways for examination of cellular and molecular pathways, we may need to inject cells or reagents in order to activate or block identified pathways in whole animals. Through such experiments we will examine the impact of pathway manipulation in thymus function and T cells development. We will administer either cells or substances via the least invasive route possible. In all cases, we will ensure to minimise any adverse effects through use of minimum possible needle size and number of injections.

In a small number of experiments, we may need to deliver cells or substances directly to the thymus (intra-thymic injection) in order to avoid introduced materials ending up at the wrong site or influencing other cells/organs if they were to be delivered for example by intra-venous injection via a peripheral vein. We have refined the technical approach to conducting intrathymic injection to ensure the surgery is less invasive by reducing the size of the skin incision to the minimum possible size and removing the need to perform a thoracotomy (incision between the ribs) or incision through the breastbone.

Tissue transplantation

In a small number of experiments, we will perform transplantation of thymus tissues under the kidney capsule of adult mice. Here, we will isolate thymus from embryonic genetically altered mice and transplant it under the kidney capsule of an adult host mouse. Importantly, due to the highly vascularized nature of the kidney, the transplanted thymus becomes fully connected to the host circulatory system and is able to operate as a 'normal' thymus over an extended period of time. Using this system, we are able manipulate thymus tissues in vitro (for example remove or add a particular cell type in the thymus) prior to transplantation. Essentially this allows us to create a 'designer thymus' in vitro. We can then transplant the donor thymus tissue into a whole animal model in order to study its development and function. This approach allows us to reduce the number of complex genetically- altered mice that need to be bred due to our ability to generate manipulated thymus tissues in vitro. In all experiments, tissue transplantation is performed solely under the capsule of a single kidney.

Importantly this refinement helps to mitigate any risk of perturbed kidney function, due to the unoperated kidney being able to provide sufficient function in the very rare event that surgery compromises function of the operated kidney. We continue to seek to refine such approaches, for example we have previously sought to identify less invasive sites for tissue transplantation, such as under the skin of the ear.
However, this has so far met with limited success and reproducibility for thymus transplantation potentially as a result of the degree of blood flow at the site of transplantation. However, we will continue to explore opportunities to refine this approach wherever possible. Following surgery, mice will be placed in warming chambers and closely monitored post-surgery to ensure that mice display appropriate recovery and behaviour, with animals subsequently being maintained for the minimum possible amount of time to achieve experimental objectives.

Why can't you use animals that are less sentient?

In this project we will use mice as an experimental model. The mouse is the species of choice as it supports the production of embryos at carefully staged points of development. Embryonic mice will be used as a source of cells and tissues (including thymus) to perform in vitro organ culture experiments that allow us to both reduce and inform our work in mice at later stages of life. The mouse replicates the mammalian placental pattern of human development over a gestation period of 21 days and provides the closest model that replicates the development, maintenance and loss of thymus function with increasing age. The mouse is also the only species that provides a wide range of natural and induced mutants with defined genetic alterations that allow the study of target molecules crucial to the function of the immune system. A large body of published data has been generated characterising the immune system in murine models, and the vast majority of knowledge regarding thymus development and function is solely based on the use of murine models since the first formal description of the critical role of the thymus in T cell development in the 1960s using mice as an experimental model. Importantly, the use of species that are less sentient remains limited. For example, Drosophila fly models do not possess an equivalent of the lymphoid lineage, including T cells nor do they possess a thymus which is evolutionarily restricted to jawed vertebrates. While fish models e.g. zebrafish possess a thymus, notable differences preclude their use as a model to fully replace the use of mouse models, particularly in regard to studying thymus biology in relation to bone marrow transplantation due to these animals lacking bone marrow haematopoiesis with blood cell development taking place in the kidney of adult fish. Although this project will use embryonic models wherever possible, we will however need to extend use of animals to adult stages in order to track postnatal stages of thymus development and maintenance that include critical stage specific roles for the thymus in T cell production.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our research group has played a significant role in refining approaches to study thymus development and function in mouse models that have helped to reduce the number of animals used in in vivo studies. For example, we have played a major role in developing novel in vitro models for the study and manipulation of thymus tissues. Such approaches have been widely adopted within the international research field and have provided an important model to validate candidate cellular/molecular pathways without the need to use complex in vivo models.

Collaborative work by our research group has additionally sought to refine approaches for the ectopic engraftment of lymphoid tissues in murine models. In this approach, we have sought to refine methods for applying the least invasive surgical approach to transplant lymphoid tissues into adult host mice. We will continue to seek to refine in vivo surgical approaches and continue to ensure that only the most robust and reproducible, yet least invasive approaches are used and this will continuously be assessed on an experiment by experiment basis.

We will always aim to keep our experiments to the minimum possible duration to meet our experimental end-points, and will always use the fewest possible interventions (e.g. injections) that are needed. Best practice in animal husbandry e.g. handling and enrichment will also be adhered to.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our experiments will be planned, conducted and reported in line with the PREPARE (planning) and ARRIVE (reporting) guidelines. We will also make use of the NC3Rs Resource hub e.g. 'Breeding and colony management' and 'Experimental Design' to inform our approaches. LASA guidelines will be adhered to and used where appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to make good use of NC3Rs resources both through the NC3Rs website, and also at a local level via communications and activities driven by the local 3Rs Focus Group e.g. regular distribution of the NC3Rs newsletter. We will seek to engage with relevant seminars, conferences and webinars where available, and will maintain contact with NACWOs, and the NVS where necessary. As described elsewhere, we will also continue to review the scientific literature to ensure that we are able to identify and potentially adopt any new approaches and/or models that may supplement or replace our current animal models.

126. Cancer Drug Discovery

Cancer Drug Discovery

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Immunology, Therapy, Immuno-oncology, Oncology

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's the aim of this project?

Our Company's overall aim is to provide a suite of advanced, mode-of-action models that will allow pharmaceutical and biotechnology industries to test novel and existing drug candidates for use in the field of oncology and immuno oncology. In vivo models are combined with advanced in vitro assays that allow us to study the effects of drug candidates.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
This project aims to accelerate drug discovery in oncology and immuno-oncology at a time

when there are exciting developments in the field of cancer therapy and immunology. Although cancer therapies are improving there are still significant gaps in treatments available.The short-term benefits from this project will be that our expertise in immunology and drug development will be available to clients lacking our level of expertise, thereby facilitating the evaluation (chiefly efficacy and mechanism of action (M-o-A), but with some safety data also obtained) of potential new products, improving the go/no- go decision making process. The long-term benefits of our work in this area, collaborating with a range of organisations, are therefore expected to be: a) new and improved drug regimes for a range of cancers; b) better clinical management of cancer and c) societal benefits from lower disease burden and greater productivity.

What outputs do you think you will see at the end of this project?

We provide a suite of advanced, mode-of-action models that will allow the pharmaceutical and biotechnology industries to test novel and existing drug candidates for use in many conditions where the immune system plays a role. Information from studies performed are used to make decisions about the progression of drug candidates to clinical trials.

Who or what will benefit from these outputs, and how?

In the short term, these outputs will assist the pharmaceutical and biotechnology industries in making key decisions about their drug discovery pipelines and whether to progress a drug candidate towards clinical trial. In the long term, this benefits patients as it prevents exposure to ineffective therapeutic agents and also facilitates the development of novel drug candidates that may have substantial clinical benefits.

How will you look to maximise the outputs of this work?

As work is performed solely on a commercial basis, for biotech and pharmaceutical clients, output will be subject to client confidentiality and therefore collaborative opportunities will be limited and at the discretion of the client, however, some of our previous clients have published our data in peer reviewed journals and presented at Scientific conferences. As dissemination of information may not be feasible, we will maximise the usefulness of the output through high quality experimental design and review of proposed experiments for scientific merit before proceeding with any work.

Species and numbers of animals expected to be used

- Mice: 6550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to replicate human disease, we shall predominantly use adult mice, with an intact, mature immune system in this project, but may also use other stages if required. The role of the immune system and the mechanisms of disease in these models closely mimic a lot of the aspects observed in the human disease. Therefore, use of these animals for drug development is directly relevant for the clinical treatment of humans.

Typically, what will be done to an animal used in your project?

It is envisaged that many of the genetically-modified animals will be humanely killed to provide cells and tissues for use in the laboratory and will undergo no further experimentation. Where experimentation is conducted, procedures include assessment of cancer models (where tumours are induced in healthy animals), dose finding procedures and the analysis of the process by which healthy cells are transformed into cancer cells (compound-driven carcinogenesis). All animals will be humanely culled at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that have received a subcutaneous cancer cell transfer will experience tumour growth, this will normally be in the flank and should induce minimal direct adverse effects. However, to replicate the effects of human cancer, listed models may lead to clinical signs including tumour ulceration, which is normally not accompanied by bleeding, exudation, or infection. Animals will be closely monitored throughout for signs of distress and culled humanely if they exceed the predefined limits.

Tumour location, onset of ulceration, bleeding, exudation, and other animal welfare observations will be recorded in detail and may allow further refinement of the models. If the study goals are achievable prior to reaching the tumour size endpoint, this will be reflected in earlier termination. This option will be agreed with the client at the study design stage, with consideration given as to the best way to gain all necessary data from each experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of the animals used will experience mild (60 %) to moderate (40 %) effects. Direct effects from tumour growth should be mild, with other animals experiencing mild discomfort following administration of treatment.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?
Importantly, many of our assays are performed in the laboratory, rather than in animals. These often use human cells and allows us to test how different cells respond to potential drugs. However, once we have defined the most likely candidate drugs, we must understand whether these can in fact help to promote the immune response seen in cancer, ultimately reducing tumour size and improving long term prognosis.

Which non-animal alternatives did you consider for use in this project?

We routinely perform in vitro assays using human cells in order to test drug candidates on cells of the immune system. The majority of our work is conducted using these in vitro assays, however, animal studies are often required downstream of this in order to test drug efficacy in a living system prior to clinical trials in humans.

Why were they not suitable?

In vitro assays are very useful at measuring the effect of drug candidates on particular cell populations and complex assays can be designed to model a lot of the processes that occur in the body. However, in vitro systems currently cannot model all of the aspects involved in cancer that occur 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?

This estimate is the maximum number of animals predicted. As a CRO it is difficult to accurately calculate the exact number on animals that will be used as this largely relies upon client requirements and demand. Whilst we offer in vivo models as a service, these experiments are ordinarily only performed as a small part of a larger package of work that is primarily focused on in vitro models.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our emphasis as a company is to use data-rich in vitro methods so that clients can select the best compound(s) before moving forward to in vivo models. This means that drugs that are unlikely to be effective are screened out at an early, in vitro stage. We have almost 5 years ' experience with these models and as part of establishing these models, we optimised the models so that we could use as few animals as possible but still obtain sufficient relevant information.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
Our advanced in vitro assays allow us to advise our clients on which of their test compounds are most likely to be effective in in vivo studies. This saves time and money and, most importantly, reduces the numbers of animals required for studies.

In addition, pilot/initial studies will be performed using the least invasive protocols and only using drug candidates that have been proven to be effective in in vitro experiments. We will also apply statistical procedures to determine that we are using sufficient but not excessive numbers of animals per group for the optimum scientific design.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our objective is to provide advanced, mode-of-action models that will allow the pharmaceutical and biotechnology industries to test novel and existing drug candidates for use in cancer. In vivo models are combined with advanced in vitro assays that allow us to study the effects of the test drugs on tumour growth during the development of cancer.

Many of the genetically-modified mice used within this project will be humanely killed to provide cells and tissues for use in the laboratory and will undergo no further experimentation. Beyond our breeding program, the majority of the WT and GM animals used will experience mild to moderate effects. These include administration of inflammatory agents to provoke an immune response.

In some experiments we will also need to induce tumour growth so that we can further study the immune response in a cancer model, or to analyse the effect of treatment upon tumour growth. We now have experience with several of these models and animals will be closely monitored throughout to minimise pain and suffering.

Why can’t you use animals that are less sentient?
Many of our clients begin their programs of work using isolated cells from rodents from which sufficient quantities of desired cell types expressing a target of interest can be obtained in sufficient quantities for moderate throughput, data rich in vitro screening assays. Those successful "Hit" compounds can then be further progressed to rodent in vivo models to ensure continuity of species used. Rodent models are extremely well-characterised and validated for studying the progression of cancer and for evaluating the efficacy of new therapeutics. They also have the most extensively characterised immune systems, which is relevant for understanding the role immune cells play in the progression of cancer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?
Where necessary, animals will receive appropriate anaesthesia during the procedure and analgesia following surgery. In some experiments animals are at risk of infection. These animals will be kept in a barrier environment and receive appropriate antibiotics. All animals are monitored regularly and any that show signs of ill health receive prompt veterinary intervention. If significant ill health is evident, the animals are humanely culled. Any further refinements that can be implemented over the course of this projects will be put in place following consultation with the Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS).

Subcutaneous tumours will be monitored twice weekly by caliper measurement, with those animals reaching 15mm diameter being culled to minimise any adverse effects. Tumour

location, onset of ulceration, bleeding, exudation, and other animal welfare observations will be recorded in detail and may allow further refinement of the models.

The vast majority of animals that receive intraperitoneal, or intravenous, administration of neoplastic cells will not show any clinical signs of tumour growth. Animals will be closely monitored and any showing any adverse effects will be humanely culled.

In terms of general handling, as advised by our establishment we have adopted non-aversive handling methods where feasible and update our practices according to recommendations.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to following the PREPARE guidelines and the best practice procedures recommended by the NC3Rs, regular literature searched will be performed to ensure that the models used are the most refined for each purpose.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

In addition to communications and seminars from the University Veterinary Services, we will routinely access the resources on the NC3Rs website (https://www.nc3rs.org.uk/) and subscribe to the NC3Rs newsletter in order to keep up to date with current developments.

127. Characterising mechanisms involved in Gastrointestinal diseases

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immune System, Metabolism, Blood vessel development, Gastrointestinal disease

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, juvenile, neonate, adult, pregnant
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to identify cell populations and mechanisms involved at key stages of gastrointestinal disease progression. We will develop and assess new treatments to stabilise, or reduce gastro- intestinal disease progression and also novel diagnostic tools.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Gastrointestinal diseases, such as inflammatory bowel disease, affect 449 patients per 100,000 people in the UK. Patients with inflammatory bowel disease experience abdominal pain, severe diarrhoea, fatigue, weight loss and malnutrition. In more serious cases surgery is required to remove the affected bowel area.

Approximately 33% of patients do not respond at all to limited current therapy offered and a further 50% lose response over time. Persistent inflammation in the gut can lead to cancer. Colorectal cancer is the third most common cancer in the UK, with only 53% of patient with colorectal cancer surviving for more than 10 years.

Gastrointestinal diseases are classed as multi-factorial diseases. This is because there are many factors and cell populations that initiate and cause progression of gastrointestinal disease to advanced stages. However, the mechanisms that drive gastrointestinal disease are not fully known. Neither are the associated mechanisms and cell populations involved in the lack of response or resistance to current therapy.

It is important to be able to continue to identify these mechanisms. Using this new knowledge, we can develop appropriate therapy to reduce, stabilise and reverse gastrointestinal disease. Collection of samples at key stages will enable us to develop biomarkers to identify disease stage, and predict outcome and response to therapy.

What outputs do you think you will see at the end of this project?

Using mice and zebrafish to model aspects of gastrointestinal disease (including the use of syngeneic models and genetic modified animals) we will have gained the following knowledge at the end of this project:

Improved knowledge of how different aspects of the immune system and other cell types (such as microbiota or stromal cells) and factors are involved in aberrant biological processes, which drive intestinal diseases.

Improved knowledge on how diet can influence initiation and progression of gastrointestinal disease.

Improved knowledge of the different cell types of the gut respond to low oxygen and nutrient levels in normal and pathological states within the gut.

Identify mechanisms of resistance to current therapies used in gastrointestinal disease.

Developed new treatments (including therapeutic antibodies) to reduce, stabilise or reverse gastrointestinal disease.

Developed biomarkers (including diagnostic antibodies) to identify disease stage, and predict outcome and response to therapy.

This information will be shared with the scientific community through publications, conferences and online data portals.

Who or what will benefit from these outputs, and how?

Patients with gastrointestinal diseases, clinicians and academic researchers will benefit from the outputs of this project.

Ultimately, the results of this research will enable improved diagnostics for patients and also inform which treatments are most appropriate in gastrointestinal disease. This project will enable us to have a better understanding of the mechanisms and involvement of cell types that hallmark different disease states, enabling improved drug targeting in patients.

How will you look to maximise the outputs of this work?

This information will be shared with the scientific community through meetings, publications (including unsuccessful approaches involving animals) and conferences. In addition, we hold in regular person meetings and invite collaborators from diverse scientific backgrounds to attend and exchange knowledge and experiences. These improve the outputs of our projects.

Species and numbers of animals expected to be used

- Mice: 5000
- Zebra fish (Danio rerio): 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.

Zebrafish: We will be using zebrafish as the lowest organism in which gut, vasculature, metabolism, immune development is well studied. Some of the cell types involved in these processes in humans have not been identified in mice; for example, those involved in gut formation. Zebrafish need to be at the age that they can bred and produce healthy offspring. Studies on development and factors involved in gastrointestinal disease would be conducted up to the stage of young fish, when the immune system is functional.

Mice: We will be using mice to model the complex interaction between cell types and regulation of processes that occur in humans and generate clinically relevant information. Mice need to be at the age that they can bred and produce healthy offspring. Successful modelling of gastrointestinal disease and evaluation of therapy is dependent on the use of young adult mice. Juveniles, neonates and pregnant females are used in cases where early deletion of a gene is required, based on the expression profile of that gene.

Typically, what will be done to an animal used in your project?

In Zebrafish: Genetically altered zebrafish may be bred and given an injection of a gene inducing or deleting agent to modify its genetic status. The administration of these drugs will be typically for a time ranging 72h.

Embryos are given an injection of a gene inducing or deleting agent to modify its genetic status once.

Fish may be injected with cells once, continuously exposed to microbiome, or exposed to substances to evoke elements of gastrointestinal disease stages. The administration of these substances can be continuous in water or food for eight weeks.

Fish may be continuously exposed to therapeutic microbiome or substances in water or food for eight weeks to modify the process which is being studied.

Developing embryos or fish may be exposed to substances and imaging to visualise changes in during key developmental and disease stages.

Samples will be collected at key development or disease stages to assess changes (e.g. for ex vivo assessments).

In mice: Genetically altered mice may be bred and given an injection of a gene inducing or deleting agent to modify its genetic status. The administration of these drugs will be typically daily for one week.

Mice may be given a substance or cells (such as immune cells or microbiota) to modify the process which is being studied. Administration of substances or cell types/microbiota to initiate gastrointestinal disease. The administration of these substances typically being repeated cycles of daily treatments for one week, followed by rest period until stage of gastrointestinal disease is reached.

Administration of substances or cells to modify gastrointestinal disease progression as a single or combination of treatments.

Imaging or endoscopy may take place to identify changes that occur at key disease stages.

Typically, samples will be collected at key disease stages to develop biomarkers or therapeutic antibodies and to assess changes in gastrointestinal disease.

What are the expected impacts and/or adverse effects for the animals during your project?

Zebrafish may develop abnormally, display small signs of internal bleeding, signs of redness to indicate inflammation, become lethargic, may fail to maintain weight, have signs of diarrhoea, or become constipated.

Mice with gastrointestinal diseases will be expected to develop diarrhoea and experience slow weight loss.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Zebrafish: Subthreshold (10%), Mild (40%) and moderate (50%)

Mice: Subthreshold (40%), Mild (26%) and moderate (34%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal disease models, in combination with imaging, will allow us to study closely the cellular mechanisms of initiation and progression of gastrointestinal disease. We will also be able to assess the effectiveness of the new diagnostics and therapies. These can be translated to the clinical setting.

The animal models of gastrointestinal disease mimic aspects of the complex processes and interactions between cell types that lead to the disease, or cause progression of the disease. Some of these processes involving immune cells and other cell types can be studied in lower organisms such as zebrafish.

Which non-animal alternatives did you consider for use in this project?

Access to material and results from patients with gastrointestinal disease in the clinic and previous animal experiments are available and will reduce the number of animal experiments required. Collectively, this material and data can be interrogated using computational approaches to aid finding new links to processes and mechanisms driving gastrointestinal disease progression.

We can model certain basic processes in the laboratory setting. Cell culture will be used throughout this project to provide key information on gene function and mechanisms of cell adaptation to specific stresses in culture. We can model basic outcomes following exposure to new therapies (such as reduce rate of disease progression).

Why were they not suitable?

At present, the use of non-animal methods does not allow us to answer all of our scientific questions. Non-animal models cannot fully recapitulate the 3D nature of the gut tissue architecture and the many different cell types interacting with each other, the exposure to microbiome, fluctuations in blood delivery of oxygen and nutrients and how these collectively influence response to therapy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 have shown what number of animals are required for each study and conditions investigated with appropriate numbers of animals and controls in the experimental design. Data on the numbers of animals required for breeding and maintaining a productive colony is also available from our archive of animal database.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We regularly cross-reference our experimental design, where appropriate with online tools such as the NC3Rs Experimental Design Assistant. We also use statistical design models to aid confirmation of appropriate numbers of animals to be used.

Computational analysis of non-animal model data, previous animal experimental data and information from pilot animal studies also aids the selection of appropriate targets and experimental design to improve the quality of data obtained at the end of the experiment but using the least number of animals. An example of this is incorporating imaging points into our design. Use of imaging increases the output from each animal, creating a better picture of the changes that occur at key disease progression stages without the need to kill animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Well planned, efficient breeding calculations are carried out to ensure that only those animals needed for colony maintenance and experimental studies are produced. An animal database gives us the most recent data to enable these calculations to be carried out.

The use of imaging techniques allows for more information to be gathered from each individual animal.

Harvesting of extra tissues and appropriate sample collections at the end of the experiment and banking them allows for future use and negates the need for animals in these cases.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals lacking a functional aspect of the immune system will be used to investigate which part of the immune system initiates and cause progression of gastrointestinal disease.

We will use animals with or without a genetic change because we can directly study the mechanisms involved in specific processes in disease progression, for instance blood vessel development or metabolism changes, response to microbiota presence. Use of genetic modifications enables targeting specific key pathways/mechanisms that is impossible to replace by an alternative method, e.g. due to current lack of appropriate specific inhibitors and avoids animals being subjected to multiple administration of substances. Animals used for breeding will only undergo methods that are natural and suitable to maintain the genetic status of the strain.

Pilot studies are always conducted using the fewest number of animals to establish the profile and outcome following modifications or exposure to novel substances/cell types.

Imaging provides important insights into disease progression, enabling us to visualise stages of disease (e.g. extent of inflammation and damage to gut lining) and real time changes inside the gut without dependency on changes in animal health and behaviour.

Why can’t you use animals that are less sentient?

The experiments proposed are being conducted in the least sentient possible animal system.

Zebrafish: In this project, we will be using zebrafish, being the lowest organism in which gut, vasculature, metabolism, immune development is well studied during the first five days where animals do not feel pain. Zebrafish lay their eggs externally and therefore we can easily collect embryos without harming the breeding females.

Embryos and free-feeding fish are required to study developmental stages and aspects of disease initiation. The majority of experiments will terminate before the free-feeding stage is reached. Only zebrafish models required for observing modulation of the immune system will end after eight weeks.

Through the use of zebrafish, we can learn and identify new cell types and methods involved in developmental stages and also in controlled initiation of disease states. Some of these cell types are not found in mice but are in human; for example, those involved in gut formation.

Mice: Juveniles, neonates and pregnant females are used in cases where early deletion of a gene is required, based on the expression profile of that gene.

The adult mouse model is relevant to our studies because they enable us to address our studies into complex processes involving multiple cell types that promote gastrointestinal disease initiation and progression. The mouse model is appropriate to study gastrointestinal disease due to the need to study the different functions of the immune system that are not present in the zebrafish.

Initiating gastrointestinal disease in animals, exposure to cells (immune, microbiome) and substances (diagnostic, therapeutic) are being conducted in mice at an appropriate age, using well studied mechanisms and models of gastrointestinal disease.

The mouse model is most widely used and accepted animal system for these types of studies. In addition, the information provide at this animal stage and disease models translates into the clinical setting.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experimental designs are based on previous recorded and pilot experimental observations and outcomes. This ensures appropriate use of procedures and routes to minimise welfare costs for animals. We provide appropriate pain management following any procedures.

We regularly monitor animals for changes in their health, for example measure their weight change and their behaviour.

Observations during experiments are documented to allow control measures such as increased monitoring or treatments to reverse, reduce or prevent further harms to the animals.

We always conduct pilot studies to check that measures and experimental designs will minimise welfare costs for animals when necessary for novel substances/cell types.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The design of experimental procedure will be compared with best practices guidance in order to ensure the most refined methodology is used, such as: local AWERB guidelines, those outlined on NC3Rs (e.g. ARRIVE guidelines), Guidance on Animal Testing and Research from the Home Office, LASA and RSPCA guidelines, PREPARE guidelines and published guidelines on gastrointestinal disease models.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly review publications (including those relating to the 3Rs) and attend conferences, discuss with other experienced groups, Named Animal Welfare Officer, hold in vivo meetings to ensure that we constantly implement advances in techniques and non-animal alternatives. We also check experimental needs and designs with information on websites, such as Home office, the NC3Rs, LASA and RSPCAs.

128. Studying how signalling proteins control development and regeneration in zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Embryogenesis, Organ function, Wound healing, Regeneration, Growth factor signalling

Animal types	Life stages
Zebra fish (Danio rerio)	adult, neonate, 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's the aim of this project?

Our aim is to understand how two families of growth factors, namely Wnt and Shh, controls fundamental cell behaviours, namely proliferation, differentiation, and migration during complex tissue formation in the early embryo. In addition, we want to understand the roles played by these signalling systems during wound healing and regenerative processes in the fin, heart, and brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We still do not understand how growth factors regulate fundamental processes like organ formation and wound healing. We envisage that this programme of work will lead to an improved understanding of the genetic and cellular processes involved in cell proliferation, differentiation, and migration leading to organ formation and tissue repair. This is important

because we will then understand the beneficial functions of growth factor signalling during embryogenesis and regeneration.

Zebrafish have a remarkable capability to heal and regenerate many tissues, specifically the fin, heart, and brain after injury. We will decipher these signalling mechanisms to identify mechanisms and treatments that could be used to promote wound healing in humans.

What outputs do you think you will see at the end of this project?

We envisage that over the 5 years of the programme, we will learn new knowledge of the genetic and cellular processes regulated by the growth factor signals Wnt and Shh, leading to organ formation in embryogenesis and wound healing. We envisage three key outputs during the project:

Identification of mechanisms regulating cell proliferation, differentiation and migration guided by Wnt and Shh signalling in embryogenesis

Identification of essential proteins regulating these signalling cascades controlling fundamental decisions in regeneration

Identifications of genes and pharmaceutically active compounds influencing signalling process

The primary way we will communicate our findings is through peer-reviewed publications in specialist and generalist journals. We will also present information orally at scientific conferences and to the general public through public engagement talks.

Who or what will benefit from these outputs, and how?

Human health. Although this research project is generally in the area of basic biology, it has the potential to impact human health. Over 70% of all human genes have at least one zebrafish equivalent (ortholog), making our work potentially relevant in the vertebrate lineage. The evolutionary conserved Wnt and Shh signalling pathways regulate both the maintenance and differentiation of all cells in the body, including stem cells. Growth factor signalling is activated within minutes after tissue damage and fulfils a vital role for protection in cell survival. Signalling is a prerequisite for tissue repair from zebrafish to humans: For example, when the heart muscle is damaged, a functional heart will regenerate, provided that Wnt and Shh signalling is active at the time of damage. However, if it is not active the result is an incorrectly functional heart with scarring. In mammals, including humans, blockage of Wnt and Shh signalling delays wound healing and leads to insufficient wound closure.

Developmental biology. Our insights into how the signalling behaviour of individual cells is regulated in a tissue context in the living vertebrate organism will advance our understanding of the mechanistic basis of cell-cell communication. In contrast to passive diffusion, controlled dissemination of signalling molecules will open up a new field to control of signalling range in a growing tissue. The far-reaching importance of this finding is already apparent as these were recently discussed in a chapter of the latest edition of Scott Gilbert's 'Developmental Biology' textbook for pupils and students.
Signalling biology. Our results will provide insights into the relationship of signalling in individual cells. This will advance the field since most of our knowledge of signalling comes from the analysis of simple in vitro systems or invertebrate model organisms. Wnt

and Shh signalling regulate the progression of tumours, and it has been shown that 95% of patients with colon cancer have a massively increased production of these growth factors in the tumour tissue. Also, growth factor signalling is strongly down-regulated in Alzheimer's diseased brains leading to neuronal death. Advanced systems allowing more direct insights into cellular signalling by studying growth factor trafficking in a living vertebrate animal are currently limited. Our experiments will serve as a novel basis for manipulating signalling in diseased tissues.

Cell biology. In recent years, due to live-cell imaging, evidence is mounting that the cytoskeleton is highly dynamic and controls many processes beyond cell shape changes. The proposed analysis of cell protrusion formation in zebrafish embryos will yield valuable insights into this fascinating field of cytoskeletal dynamics, also contributing a novel perspective on the regulation of these processes by signalling cascades.

Quantitative biology. New quantitative approaches for describing molecular relationships from sub- cellular imaging are critical for developing the field of cell and developmental biology. Throughout the proposed study, we will develop and refine tools to quantify signalling molecule concentration in a tissue required to activate the signalling cascade.

Zebrafish biology. This work will further develop the early zebrafish embryo as a model system, particularly highlighting the benefits of the cells of the embryonic ring as a source tissue for various signalling molecules that is well suited for studying the cellular signalling process in vivo.

In summary, insights into the mechanisms underlying the regulation of signalling gained during this project will increase our knowledge of two of the most important signalling cascades, Shh and Wnt, acting in all multicellular organisms – from worm to human. In addition, we will substantiate our fundamental understanding of growth factor signalling during development and regeneration as a short- term goal. Therefore, we will make our results available to the scientific community through research publications and conference contributions. In the long-term, we envisage the development of drugs to tackle the cell-protective function and the injury repair process based on the knowledge generated in this project. Furthermore, our acquired knowledge on Wnt and Shh trafficking might help identify drug targets for neurodegenerative diseases and cancer.

How will you look to maximise the outputs of this work?

Wherever possible, we will share knowledge and experience in our peer-reviewed publications and will utilise the skills and expertise of colleagues at our institution and colleagues nationally and internationally to generate high-quality studies with the most meaningful data. Wherever possible, we will implement new and thoroughly developed methodologies to stay current with our procedures. This includes attending NC3Rs seminars, workshops and conferences.

Negative results will be submitted for publication or uploaded to preprint servers, where appropriate.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 28000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this programme of work, zebrafish are used as a model organism to understand growth factor signalling during development and diseases in vertebrates.

Genetics will play a major part in this project. Zebrafish have a similar genetic structure to humans and share over 70 per cent of genes with humans; over 80 per cent of genes known to be associated with human disease have a zebrafish counterpart. The zebrafish genome has been fully sequenced to a very high quality. This enables us to create mutations in these disease-related genes and study their function.

To study the growth of the vertebrate embryo, the zebrafish is an excellent model as light triggers mating in zebrafish. Then the zebrafish produce hundreds of offspring at weekly intervals leading to a great supply of eggs with an identical genotype. Zebrafish eggs are fertilised and develop outside the mother's body. This allows us to study developmental processes in a non-invasive way. In addition, the zebrafish embryo/larvae grow at an extremely fast rate allowing to study processes of cell proliferation, differentiation and tissue growth. In this work programme, the distribution and function of growth factors in a tissue will be investigated and the zebrafish embryo/larvae is nearly transparent makes it ideal to study growth factor signalling in an intact tissue by high-resolution microscopy. To complement our studies, we will also use the adult zebrafish to study growth factor function in wound healing and regeneration. As a vertebrate, the zebrafish has the same major organs and tissues as humans. Their muscle, blood, heart and brain share many features with human systems. Of specific importance to this project, zebrafish have the unique ability to repair nearly all organs - including the heart, brain and fins. For example, if a part of these organs is removed zebrafish can grow it back in a matter of days or weeks. In this project, we will work to find out the genes involved in this process to see if this will help us to develop ways of repairing damaged organs in humans.

Typically, what will be done to an animal used in your project?

Most adult animals (95%) will be kept and bred to study growth factor signalling in the embryo/larvae.

In particular, we will study the distribution and function of growth factors in the zebrafish embryo/larvae pre-independent feeding (5days post-fertilization or younger, pre-ASPA). We will use the translucent embryo/larvae to visualise the spread and the function of growth factors by various microscopy techniques.

A few adult animals (5%) will be used to study similar processes such as cell proliferation and differentiation guided by growth factors during wound healing and regeneration. Some animals will undergo surgical procedures, most taking around 15 minutes to complete, although some could be a little longer (~60 mins) and some very short (~10 mins).
To study the process of wound healing, we will remove parts of the fin tissue under anaesthetic and study wound healing, and the formation of the regeneration bud.

As a myocardial infarction/heart attack model, the fish will have a localised area of damage created on the ventricle of the heart under anaesthetic. The animal is returned to its tank and monitored for 14 days to assess the regenerative capacity of the heart.

To study neuronal regeneration, we will treat zebrafish with chemical compounds generating synaptic fragmentation and synaptic loss as observed in the Alzheimer's diseased brains in humans.

After the procedure, animals will be used for non-invasive, microscopic visualisation of regeneration processes and occasionally be treated with existing and new drug therapies in the following 90days. During the procedure, fish will be regularly monitored.

What are the expected impacts and/or adverse effects for the animals during your project?

For the breeding and maintenance of zebrafish to generate embryo/larvae, we do not expect adverse effects for the adult fish. Fish will usually be kept in holding tanks. Occasionally, fish will be transferred into breeding tanks for egg deposition overnight. On the following day, fish will be transferred back to the holding tanks.

For regeneration experiments, we expect the tissue regeneration to resemble human wound healing. For example, on the fin, healing will likely include the formation of epithelial appendages at a wound edge, resulting in scarring of the epidermis. We will use optimised procedures including analgesia to minimise adverse effects and will monitor the behaviour of the fish daily. Fish are not expected to lose their balance in the water, ability to swim or perform other normal functions. They are expected to recover within 7 days and the regeneration process, e.g. for fin or heart, is expected to be completed within 60 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Zebrafish 80% mild
20% moderate

Most animals will be under breeding and maintenance protocols undergoing no procedures with no expected adverse effects. These animals will experience no more than mild severity (<10%), many of them are likely to experience sub-threshold severity (>90%).

15% of animals will have a GA in proteins affecting Wnt or Shh signalling, which may affect their ability to swim. This may cause moderate suffering in the affected animals.

5% of animals will be under experimental protocols where wounds will be made under anaesthesia, then the animals monitored for a number of days to assess regeneration of tissue. These animals will experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The majority of our experiments are actually conducted with cells in Petri dishes using cultured cell lines. This is where we generate preliminary data to escalate to animal work, should the data support this.

However, the embryo/larvae, the fin, the heart, and the brain are complex 3-dimensional structures containing many different tissues such as neuronal tissue, muscles, ligaments and bones, subject to frequent movement. None of the 2D models resembles all aspects of cell behaviour in these complex tissues. We, therefore, need animals to understand what happens to the different cell types as a model for common human disorders such as during organ formation, wound healing and regeneration. Therefore, we will seek, review and incorporate alternative assays to replace animal experiments throughout the project duration if appropriate.

Which non-animal alternatives did you consider for use in this project?

We have searched the Fund for the Replacement of Animals in Medical Experiments (FRAME) website for alternatives to the use of animals for these studies. Possible alternatives found are listed below:

Computer modelling

Cell lines

The use of early developmental stages of protected animals

Human tissue samples

Whenever possible, we try to use established cell lines, these can be either animal or human. Ideally, primary zebrafish cells would give us a great in vitro model but these are hard to come by and difficult to maintain. Similarly, the use of early developmental stages of protected animals can be a good alternative.

Why were they not suitable?

The embryo/larvae, the fin, the heart, and the brain are complicated tissues containing several different cell types subject to frequent movement. No computational or cell culture model exists, which recapitulates all aspects of the complex cell behaviour in these tissues.

However, with increasing knowledge through the project, there is the potential for the replacement of protected animals. Therefore, we develop appropriate fish and mammalian tissue culture systems in parallel to our zebrafish work. The proportion of work with tissue culture has increased over time, and about 50% of our experiments are carried out in vitro. We will expand our knowledge on the development of complex in vitro models as we have done in the course of the former project licence.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are based on the number of projects we are currently running and the future planned projects. These include existing and planned funding applications. The number is based on the number of animals typically used for pilot studies and the specific numbers used during an investigation. For example, for our work blocking the function of signalling genes (by using gene knockouts) in adult fish, which we have been doing for several years, we use 6 animals per time point in each experimental group.

A significant contributing factor to the total animal numbers is the number of animals used for breeding genetically modified animals. We have estimated this number based on our extensive prior experience with similar studies. In our work, we mainly focus on early development. This work is performed on young embryo/larvae pre-independent feeding (pre-ASPA), allowing us to reduce the number of animals used at later stages of development. Therefore, most adult animals (ca. 95%) will be kept in breeding colonies during the course of the project, and only a small number (5%) will be used for active animal experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The outcomes of the experiments in tissue culture and on embryo/larvae pre-independent feeding serve as the basis for refined wound healing experiments.

Before animal experiments start, we use the PREPARE guidelines & checklist and liaise with the animal facility to design the experimental strategy. We further use the NC3R's research design tool, which feeds into the ARRIVE guidelines. Finally, based on the NC3R's experimental design assistant (EDA), sample size calculation is performed to define the minimum numbers required to achieve defined levels of statistical significance. For example, power calculations include 90% power at 5% significance to show a 25% point difference in any parameter.

In addition, we will do pilot experiments and use appropriate endpoints to reduce the number of animals further.

In parallel, we use computational models for zebrafish early development, allowing us to computationally alter some aspects of migration. These in silico models allow us to make better predictions about downstream signalling pathways, variability and therefore the number of animals per experiment.

Alongside the animal work during these 5 years, we propose further refining mathematical models for the zebrafish organ formation; these models will allow us to run more predictive experiments reducing the number of animals used. We have collaborated with mathematicians to generated computational models that replicate the dynamic action of tissue migration in shaping organs during development. We will share biological data from our in vitro and in vivo experiments to allow these groups to refine their computational

models to test ‘real’ organ formation in the hope that in future, we will be able to run more of our experiments in silico rather than in vivo.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All studies are carefully designed to minimise animal usage. This includes carrying out preliminary studies, e.g. in cell culture & in embryo/larvae followed by pilot studies. The results are then used to calculate the minimum number of animals needed in the full study. Furthermore, we also will use tissues from one fish for multiple purposes/ experiments, e.g. control samples will be used for heart tissue/ fin tissue etc. to optimise the number of fish.

In parallel, we will collaborate with mathematicians and statisticians and with other leading fish signalling groups worldwide, who use similar numbers to our own.

Finally, efficient breeding practices will be incorporated to reduce animal numbers. Working closely with the experienced zebrafish husbandry team, we will ensure appropriate colony management and health monitoring of all of our zebrafish lines with the aim of maximising welfare resulting in higher productivity from fewer animals. Specifically, this will also include cryopreservation of GA colonies not in active use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use zebrafish at embryo/larvae and adult stages.

Zebrafish is a widely used research model for studying regeneration; therefore, a significant amount of research comes from studies in this model.

Zebrafish as model organisms has several advantages: They form the same major organs and tissues as humans. The zebrafish genome is fully sequenced to a very high quality and over 70% of human genes have a true counterpart (ortholog) in the zebrafish genome. Specifically, important for these studies, zebrafish are able to regenerate different organs, including all fins, the spinal cord, the retina, the heart, the telencephalon, and the kidney. A further advantage of zebrafish is their translucence which enables their forming tissue to be visualised using non-invasive microscopic imaging, additionally, this species is open to genetic manipulation and has the lowest neurophysiological sensitivity of the species suitable for these studies.

Specifically, we have chosen zebrafish as the most refined animal model available and complement our studies with complex tissue culture experiments, computational modelling and experiments at the embryo/larvae stage pre-independent feeding/pre-ASPA. By using the translucent zebrafish in which we express fluorescent proteins in cells of interest we

can non-invasively watch many cellular processes in the embryo/larvae and - based on these results - investigate these processes in the regenerating tissue, allowing us to collect dynamic data with minimal surgical intervention.

Why can't you use animals that are less sentient?

The majority of our work is performed either in vitro, or on zebrafish embryos/larvae prior to independent feeding. The embryo/larvae possess a number of features that make them particularly valuable for use in assays that address growth factor function in development. However, as the project is also studying the function of growth factors in tissue regeneration there is some work that currently cannot be performed without working on adults. Therefore, we have chosen zebrafish - a model which belongs to the less sentient animals within the group of vertebrates to study regeneration.

We will always endeavour to use the least invasive approach possible to answer our scientific questions. To further minimise suffering and discomfort, the adult fish will be monitored daily and when there is any concern advice will be sought from the named veterinary surgeon and/or the Named Animal Care Welfare Officer and appropriate action taken.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At all stages of the project, we will conduct regular welfare assessments to evaluate the impact of procedures and regularly review our experimental processes with a view to minimising adverse effects without impacting scientific outcomes.

To refine the procedures in animals post-surgery, we will usually use an analgesic regimen to reduce the suffering of the animals during the wound healing process. In particular, we will give appropriate analgesia such as Lidocaine non-invasively by immersion for the heart regeneration experiments if no cross-reaction with, i.e. the drug treatment is expected.

By using non-invasive imaging of genetically modified zebrafish, i.e. fluorescent reporter fish lines, we can visualise the earliest events in signalling alterations, and organ pathogenesis before external symptoms such as behavioural changes are manifest. By doing so, we can reduce the suffering of the animals by setting our endpoints much earlier than is possible in rodents or other models.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We actively use the NC3Rs Experimental Design Assistant tool to plan our studies, which helps incorporates best practices for the 3Rs into our study design. (https://www.nc3rs.org.uk/experimental- design-assistant-eda)

We will also use the ARRIVE guidelines to inform both the study design and reporting of the result of our work for full transparency and to help others reproduce our findings. (https://arriveguidelines.org/)

Throughout the tenure of this licence, we will also keep us up to date through publications, conference attendance, collaboration and funding body interactions.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have signed up for the email newsletters from the National Centre for the Replacement, Reduction and Refinement of the use of animals in research (NC3Rs) and we will regularly consult their website and attend webinars to keep our knowledge in this area up to date.

We also actively use their Experimental Design Assistant tool (https://www.nc3rs.org.uk/experimental- design-assistant-eda) to plan our studies, which helps incorporate best practice for the 3Rs into our study design.

Finally, we will effectively implement advances in the 3R in our studies after consulting with the Named Veterinary Surgeons and Named Animal Care and Welfare Officer.

129. Brain mechanisms of listening and learning

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

auditory, hearing, brain, cortex, mice

Animal types	Life stages
Mice	adult, juvenile, neonate, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand what brain mechanisms underlie normal listening, and how those mechanisms go awry in brain disorders that affect listening ability.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many people have difficulty listening in noisy environments that cannot be explained by problems within the ear alone. For example, children with developmental disorders, elderly adults, and people with brain disorders such as schizophrenia may have normal hearing sensitivity on audiological tests but nevertheless report unusual difficulty with listening in noisy environments. This difficulty is thought to arise from problems with mechanisms of listening in the brain rather than the ear. However, brain mechanisms of listening are still

poorly understood. We seek to understand what auditory brain mechanisms underlie normal listening, and how these mechanisms go awry in brain disorders that affect listening ability.

What outputs do you think you will see at the end of this project?

This work is expected to provide new information about the biological mechanisms that enable effective listening in noisy environments. The primary expected benefit is the publication of new scientific knowledge about these biological mechanisms.

This work is also expected to provide new insight into how brain mechanisms of listening are disrupted in brain disorders such as schizophrenia, tinnitus and developmental language disorder. The primary expected benefit from this output is the publication of new scientific knowledge about disease-related disruptions of biological mechanisms.

Additional outputs and benefits are expected to include new algorithms that could be used in hearing aids or other sound-processing devices to facilitate perception of speech in noisy environments.

Who or what will benefit from these outputs, and how?

In the short term, outputs from this project will benefit other scientists seeking to understand how the auditory brain works. Additionally, the project will benefit students and trainee scientists, who will learn to conduct high-quality, clinically relevant scientific research through their involvement in the work.
Short-term outputs in the form of scientific knowledge will be published in academic journals, shared with academic colleagues through presentations at national and international conferences, and communicated to the wider society through public lectures and other public engagement events.

In the medium term, outputs from the project will contribute to development of novel tools for scientific research and new technologies for improving human hearing. For example, our recent research has generated new technologies for brain research and has suggested new algorithms for sound processing that accurately mimic aspects of auditory brain function.

Also in the medium term, the project will benefit the UK economy by helping to develop a skilled workforce for scientific research. Over the past 17 years, our laboratory has trained 6 postdoctoral fellows, 8 PhD students, and more than 20 BSc or MSc students. Many of these trainees have gone on to careers in science or technology.

Finally, in the longer term, outputs from this project will benefit people with hearing impairment or with brain disorders that affect ability to listen effectively. In previous projects, we have used mice to test new treatments for tinnitus, to identify novel auditory brain abnormalities in developmental disorders that also affect humans, and to determine the likely cause of hearing impairment in a human genetic disease associated with schizophrenia. In the future, we expect this work and current projects to help improve the design of hearing aids, cochlear implants and assisted-listening devices for people with hearing impairment or brain disorders affecting hearing ability.
How will you look to maximise the outputs of this work?

Outputs from the work will be disseminated primarily through open-access research publications. Novel research tools and software will be shared through open-access

repositories. We will also seek to maximise outputs through collaboration with clinicians and scientists worldwide, continuing and expanding our current collaborations with NHS clinicans and with scientists in the UK, USA, Germany and France.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice in our experiments because the auditory brain in mice is very similar in structure to the auditory brain in humans. Moreover, there are many excellent mouse models of human diseases available. We use both normal mice and mouse models of human brain disorders that cause listening difficulties in humans, including schizophrenia, tinnitus and developmental disorders.

Most of our experiments are performed on young adult mice, because we are trying to understand how the auditory brain works in adulthood. We occasionally perform procedures (such as induction of hearing loss) on neonatal or juvenile mice and then later study the same animals when they become adults, so we can investigate how the function of the adult auditory brain depends on hearing in childhood. We also compare auditory brain function between younger and older adult mice (always before the age at which mice develop chronic age-related health problems), to help us understand how auditory brain function changes across the lifespan.

Typically, what will be done to an animal used in your project?

The most invasive of our typical procedures is intracranial measurement of brain activity using an implant. Typically, adult mice undergo a 2-4hr surgery where we make very small holes in the skull to implant tiny screws in the skull and to insert very thin, hair-like electrode probes in the brain. We secure the implant to the skull with dental cement and close the wound. Mice with implants typically resume normal behaviour shortly after waking from anaesthesia. After a one-week recovery period following the implant surgery, we measure activity of auditory brain cells using the implant while the mice listen to sounds or perform sound-discrimination tasks. These measurement sessions typically last about 1-2 hours per day and continue for several weeks in the same animal.

In other typical experiments, we do less invasive procedures either in the weeks before the implant surgery, or instead of the implant surgery. For example, we may disrupt hearing in juvenile mice using earplugs or minor ear surgeries; we may measure overall auditory brain activity in anaesthetised mice using electrodes briefly inserted just under the skin; and we may train mice on behavioural tasks. Mice used for behavioural training may have their food intake restricted in their home cage, with additional food given as rewards during behavioural training.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice typically recover very quickly from the implant surgery, resuming normal behaviours (e.g., running on wheels) within a hour or two after waking from anaesthesia. We give painkillers and monitor post- operative recovery in the mice in the days following surgery, just as would be done for people in hospital. Mice with implants are weighed and checked daily to ensure that any signs of weight loss or malaise are quickly identified and treated. We also weigh daily any mice that are having their food intake restricted in their home cage, and we provide supplemental food in the home cage as well as food rewards during behavioural training to ensure that their weights remain within normal levels for an animal of similar age without food restriction.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Overall, for the entire project, severities are expected to be moderate for up to 40% of mice, and no more than mild or non-recovery for the remaining 60% of mice.

Most of our experiments are performed in young adult mice. Those undergoing moderate severity procedures typically experience implant surgery with recovery, behavioural training involving food restriction and food reward, or repeated minimally invasive procedures performed under anaesthesia with recovery. Those undergoing mild or non-recovery procedures typically experience behavioural training without food restriction, measurement of brain activity under terminal anaesthesia, terminal anaesthesia for histological procedures, or standard breeding procedures alone.

A small percentage of our experiments (<10%) involve juvenile mice. Typically, these juvenile mice undergo mild or moderate procedures for inducing hearing loss, such as insertion of earplugs or surgery on the middle ear. Once these animals grow to become young adults, they may experience the same procedures that other young adult mice do.

An even smaller percentage of experiments (<1%) involve comparisons between younger and older adult mice, to analyse normal age-related changes in auditory brain function. Our interest is in adult brain function across the healthy lifespan, so these experiments are always conducted before the age at which chronic age-related health problems would emerge in mice (i.e., before mice would be considered "aged").

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Brain mechanisms of listening are not yet understood well enough to allow these mechanisms to be studied in computer models alone. Moreover, simpler biological systems such as cell cultures cannot replicate the complexity of the auditory system, which

consists of multiple interconnected brain areas in addition to the ear. To understand how the ear and brain enable listening, we need to study live animals.

Which non-animal alternatives did you consider for use in this project?

We use computer models heavily in our research, to refine and reduce our use of animals. For example, we have used computer models to refine predictions about what aspects of listening behaviour will be affected by auditory brain abnormalities we discovered in a mouse model of developmental disorder. We have also used computational methods to develop technologies for measuring and analysing data from the brain more efficiently, allowing both refinement and reduction in the use of animals. We stay well informed about advances in computer modelling of auditory brain systems in the scientific literature, to be ready to use these models in place of live animals when possible. However, no computer models are yet adequate to enable us to achieve the objectives of this project without experiments in live animals.

Why were they not suitable?

Most computer models of the auditory brain are either abstract models designed to study one particular aspect of listening (e.g. sound localisation) or detailed models of one particular auditory brain area or cell type (e.g. superior paraolivary nucleus cells). There are a few ongoing efforts to develop system- wide models of sound processing within the ear and auditory brain areas, but these are still not fully validated for the earliest stages of auditory brain processing, and definitely not for the auditory thalamus and cortex. While we try to make use of such models to refine our experimental designs and reduce our use of animals, they are not suitable replacements for animal experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimates of animal numbers are based on our past records of animal use under our previous project licences for related experiments; predictions about how those numbers are likely to change over the next 5 years given advancements in experimental technologies; and experimental design considerations. For example, our animal number estimates for protocols involving intracranial measurement of brain activity have decreased relative to our previous project licences, because we are now able to obtain far more data from each animal than would have been possible 5 years ago.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We design our experiments to optimise the number of data points that can be obtained from each animal, for example by using high-density multi-electrode technology to increase the number of neurons that can be recorded from each animal during intracranial measurements of brain activity. In many of our experiments, experimental units are

neurons rather than animals, so this approach is a very effective means of reducing animal numbers. We also seek to use both male and female animals in our experiments whenever the phenotype of interest is exhibited by both sexes, so that breeding colonies can be maintained at minimal size. Finally, we reduce animal numbers by designing experiments with advice from statistician collaborators and using internal statistics expertise in the lab. The lab leader has considerable statistical training and expertise, and co-leads a major research methods and statistics module at the university.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We make heavy use of computer modelling to refine our experimental designs and to optimise the number of animals used in particular experiments. For example, we use computer models of auditory brain abnormalities we are studying to predict the likely effects of those abnormalities on animal behaviour or on measurements of brain activity. This modelling allows us to estimate likely effect sizes and therefore animal numbers required to test our hypotheses. We also routinely perform pilot studies where information about likely effect size is not readily available from our previous experimental work, our computer simulations, or the published scientific literature.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mice, including both normal mice and mouse models of human brain disorders such as schizophrenia, tinnitus and developmental disorders. Mice undergo experimental procedures such as behavioural training and/or measurement; minimally invasive measurement of brain activity under anaesthesia, for example using electrodes placed just under the skin or sensors placed on the scalp; intracranial measurement of brain activity under terminal anaesthesia; or intracranial measurement of brain activity in awake animals using devices implanted in the brain under anaesthesia. Even the most invasive of these procedures (intracranial measurement of brain activity using an implant) do not cause lasting harm or pain to the animals. Mice with brain implants typically recover rapidly from the implant procedure and return to normal behaviour such as running on wheels soon after waking up from anaesthesia.

Some of our mouse models of human brain disorders may have health problems other than the auditory brain abnormalities that we are studying. For example, BXSB/MpJ mice are the only known animal model of a newly discovered auditory brain abnormality in developmental disorders, but male BXSB/MpJ mice are also susceptible to adult-onset autoimmune disease. Our experiments are designed to avoid causing any suffering from health problems other than auditory brain abnormalities (which do not cause suffering for mice). For example, we ensure that male BXSB/MpJ mice are used in experiments before

the age at which autoimmune disease symptoms typically appear, and that any of these animals developing symptoms earlier than expected are terminated promptly.

Why can’t you use animals that are less sentient?

Our aim is to understand mammalian brain mechanisms of listening to temporally varying and noisy sounds, and how those mechanisms go awry in human schizophrenia, tinnitus and developmental disorders. Mice are the least sentient mammalian species with an auditory brain similar in structure to that of humans. Techniques for genetic manipulation in mice are also much more advanced than in any other mammalian species. Genetically altered mice bred under this protocol will make it possible for us to examine the roles of specific cell types and neural circuits in the auditory brain during normal listening with a precision that could not easily be achieved in any other mammal. Furthermore, our genetically altered mouse models of human developmental disorders (BXSB/MpJ mice) and schizophrenia (*Df1*/+ mice) are among the most refined and powerful animal models of these conditions available.

Our protocols include experiments conducted under terminal anaesthesia, which we use whenever scientifically feasible. However, it is not possible to fully understand the relationship between listening behaviour and brain activity by studying brain activity only in anaesthetised animals. Measurements of brain activity in awake animals can be related to simultaneous measurements of listening behaviour in the same animals, allowing for a significant refinement of experimental design.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will seek to refine behavioural training paradigms to avoid any need for food restriction or mildly aversive stimuli.

We already use best-practice methods for monitoring, post-operative care and pain management. We will seek to improve these methods with any new refinements suggested by NC3R or the NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We rely on information from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (www.nc3rs.org.uk) and the Laboratory Animal Scientists' Association (www.lasa.co.uk) when designing experiments. We receive regular newsletters from the NC3Rs and review the published literature regularly to stay informed about developments in best practice for in vivo experiments in mice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We monitor the NC3Rs website and receive regular updates from NC3R regarding advances in the 3Rs.

We are always seeking to implement advances in the 3Rs in our experiments. For example, we have sought to develop a behavioural training paradigm using wheel-running as a reward rather than food restriction and food rewards. This strategy for behavioural training would essentially amount to a form of environmental enrichment for the animals.

130. Safety and Quality Control Testing of Human and Veterinary Medicines for Batch Release

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Abnormal, Toxicity, Safety, Quality, Control

Animal types	Life stages
Mice	adult
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Drug Products for both human and veterinary use must be effective and safe. Their production is monitored carefully to make sure it complies with Good Manufacturing Practice (GMP). Quality control tests must be performed on samples from each new batch to comply with national and EU regulations.

Although some information on safety can be obtained without using animals, some tests must be carried out using animals to better understand how these medicines might affect the human body.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 test procedures detailed in this licence form an essential part of the data required to monitor the safe development, production, and release of medicinal products for use by consumers.

The ability to test these products ensures that they have been produced safely and to appropriate standards. These results provide satisfaction to the relevant Marketing Authorization that the tested products are safe to use by the end consumer. The prevention of the tests listed would result in the release of these products being delayed or denied. Depending on the exact nature of the medicinal product this could lead to serious health implications for the intended patients. As the nature of this work is quality control on each batch produced any prevention of testing would have an immediate impact.

What outputs do you think you will see at the end of this project?

The test procedures detailed in this licence form an essential part of the data required to monitor the safe development, production and release of medicinal products for use by consumers. The ability to test these products ensures that they have been produced safely and to the appropriate standards. The results provide satisfaction to the relevant Marketing Authorisation that the tested products are safe to use by the end consumer. The prevention of the tests listed would result in the release of these products being delayed or denied. Depending on the exact nature of the medical product, this could lead to serious health implications for the intended patients. As the nature of the work is quality control on each batch produced, any prevention of testing would have an immediate impact.

Who or what will benefit from these outputs, and how?

This work is being carried out as a service therefore the end user will benefit in that they will have access to health improving medicines.

How will you look to maximise the outputs of this work?

The work performed under this project licence is for clients with whom a Confidentiality agreement is in place. The tests are to ensure safety in batch release and the knowledge gained is not transferable to other products.

Species and numbers of animals expected to be used

- Mice: 125
- Guinea pigs: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult CD1 mice and Dunkin Hartley Guinea Pigs

Typically, what will be done to an animal used in your project?

Abnormal toxicity and absence of toxicity tests involve temporary restraint (e.g. scruffing), and the administration of a test substance via the intraperitoneal route. Typically, the tests have a duration of up to 7 days as advised in the Pharmacopoeia.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals may encounter weight loss or reduced weight gain. However, this is usually only minor.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All mice and Guinea pigs used in these studies are expected to undergo mild severity. Moderate severity may be observed in a failed result, however these tests are expected to pass.

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 methods listed are specifically required by legislation and/or regulatory authorities to confirm safety of the product(s), leaving little opportunity for non-animal methods. It is not possible at present to reproduce in-vitro the complex composite whole-body response produced in vivo and the availability of validated alternatives for regulatory use is somewhat limited.

The Abnormal Toxicity Test has been removed from the European Pharmacopoeia. It is still in the United States and other reginal Pharmacopoeias. The use of ATT as a method to identify potentially harmful batches and contaminants has been questioned and modern pharmaceutical manufacturers have appropriate quality control in place preventing the risk of contamination. These improved quality control measures are seen to be suitable for ensuring product safety rather than testing.

In some ROW territories where the release of a pharmaceutical won't be accepted without an ATT or GST then a case may be made to perform the test in order to allow the end user access to essential medicine.

Which non-animal alternatives did you consider for use in this project?

There are currently no non-animal alternatives for an abnormal toxicity or absence of toxicity test.

Why were they not suitable?

There are currently no non-animal alternatives for an abnormal toxicity or absence of toxicity test.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Testing at a frequency of five assays per year using five mice and two guinea pigs each time (for duration of licence, five years).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design is defined in the Pharmacopoeia.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The tests procedures are closely defined and must be carried out to a strict protocol for compliance with the requirements of the various regulatory authorities and there is therefore little opportunity to reduce animal numbers used in any specific procedure.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal to be used in these studies have been established over many years and found to be appropriate to monitor the safety of the product for use by the consumer. Historically mice have been chosen as the lowest mammal in the hierarchical level of testing where whole body response must be monitored as in Abnormal Toxicity (Safety) tests and absence of toxicity tests of chemicals. Guinea pigs have been identified for some tests where biological materials are used as they produce a similar immune response (for example an anaphylactic response) to humans.

Why can’t you use animals that are less sentient?

The animal models required for these tests are described in the pharmacopoeia monographs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Dosing and observation of the animals will be performed by fully trained staff. The animals will be monitored on a daily basis once dosed and any adverse effects reported directly to the PPLh and NACWO.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3R's are used to keep up to date with the latest science technology, new approaches, and the best experimental designs. The NC3R's also provide guidance on the latest knowledge to improve laboratory animal welfare.

Being a member of The Institute of Animal Technology (IAT) also advances knowledge and promotes excellence in the care and welfare of animals in science.

Harm-Benefit Analysis Process, Code of practice for the housing and care of animals bred, supplied or used for scientific purposes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The pharmacopeia updates are reviewed annually for any changes to specific monographs. Other changes are not always possible without changing the scope of the test and therefore it's acceptability to the receiving authority.

131. Pesticide metabolism and residues in livestock

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Pesticide, Residue, Feeding, Metabolism, MRL

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	adult
Cattle	adult, juvenile
Goats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To measure pesticide levels in animal tissues and other matrices (e.g. milk and eggs) after intake of pesticides and their metabolites.

To generate data to be used in assessing the dietary risk of pesticides to humans from consumption of meat, milk and eggs and to generate data to be included in dossiers submitted for registration of plant protection products.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Livestock residue and metabolism studies are required under various global directives including; EC Council Directive 91/414/EEC, US EPA (OPPTS 860.1480 Residue Chemistry Test Guidelines Meat/Milk/Poultry/Eggs) and Australia (APVMA Residue Guideline No. 1 – Animal Transfer Studies), when residues of a pesticide are expected to

be present in livestock feed and might cause detectable residues in livestock. The conduct of the studies, including minimum numbers of animals, dose levels and duration, is specified in guidance documents.
The use of live animals is needed in order to determine transfer of pesticide and/or their metabolites into meat, milk and eggs. Farm animals (cows, hens, etc) are required by the regulator as the objective is to measure potential dietary exposure to pesticides in their edible tissues. The minimum number of animals required is specified in the relevant guidelines and the minimum number will be used wherever possible. In some cases, the use of one particular type of animal removes the need to generate data for other animals. For example, in most cases the results of a cattle feeding study can be used to establish tolerances in goats, pigs, horses and sheep. Similarly, data on chickens is normally accepted in lieu of data on other livestock poultry, e.g. turkeys, geese and ducks. This licence allows the use of farm animal species (cattle, goats and poultry) to achieve the regulatory requirements.

All planned studies are assessed by an ethical review committee to determine if the study can proceed and all studies will be subject to ongoing review. Studies will be undertaken under commercial husbandry conditions and according to the relevant Defra Codes of Recommendation for the Welfare of Livestock.

What outputs do you think you will see at the end of this project?

Livestock metabolism studies fulfil several purposes:

Provide an estimate of total residues in the edible livestock commodities, as well as the excreta.

Identify the major components of the terminal residue in the edible tissues, thus indicating the components to analyzed in residue quantification studies (i.e., the residue definitions for both risk assessment and enforcement).

Elucidate a metabolic pathway for the pesticide in ruminants and poultry. − Provide evidence whether or not a residue should be classified as fat soluble.

The primary purposes of the Residues in Livestock study are to provide the basis for establishing maximum residue limits (MRLs) and for conducting dietary intake assessments for consumer safety.

Who or what will benefit from these outputs, and how?

Metabolism in Livestock studies are used to determine the qualitative and quantitative metabolism and/or degradation of the active ingredient resulting from pesticide use in feedstuffs, direct application to livestock, or premise treatment. Metabolism in Livestock studies are generally carried out on lactating ruminants and poultry.

Residues in Livestock studies are conducted in order to quantify levels of residues in meat, milk, eggs and edible meat by-products, such as fat, liver, kidney of ruminants, following the use of a pesticide product. The situations to which such studies apply include application of a pesticide to raw agricultural commodities (RACs), and the feeding or grazing of such commodities and their by-products by livestock; pesticides that may be directly applied to livestock; and pesticides that are used in livestock premises.

How will you look to maximise the outputs of this work?

As we are a Contract Research Organisation most studies provide accurate data which is acceptable to regulatory authorities. The data is likely to be included in dossiers submitted to the regulatory authorities when registering plant protection products. This data will enable accurate decision making with regard to labelling of the chemicals, for example.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 860
- Cattle: 300
- Goats: 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.

The use of live animals is needed in order to determine transfer of pesticide and/or their metabolites into meat, milk and eggs. Farm animals (cows, hens, etc) are required by the regulator as the objective is to measure potential dietary exposure to pesticides in their edible tissues. The minimum number of animals required is specified in the relevant guidelines and the minimum number will be used wherever possible. In some cases, the use of one particular type of animal removes the need to generate data for other animals. For example, in most cases the results of a cattle feeding study can be used to establish tolerances in goats, pigs, horses and sheep. Similarly, data on chickens is normally accepted in lieu of data on other livestock poultry, e.g. turkeys, geese and ducks. This licence allows the use of farm animal species (cattle, goats and poultry) to achieve the regulatory requirements.

Typically, what will be done to an animal used in your project?

Metabolism studies (OECD 503)

The guideline indicates that metabolism studies should be conducted using radiolabelled test compound.

The guideline recommends that the dose is administered orally via a dosing gun, capsule or gavage. Ruminants should be dosed daily for at least five days and poultry daily for at least seven days.
However, if a feeding study is not to be carried out, the dosing period may need to be extended so that a plateau of residue is reached.

A ruminant metabolism study can be carried out on one animal, whereas for poultry a minimum of 10 birds per dose level is recommended. For ruminant studies a companion animal will always be sourced.

Milk and eggs will be collected in the normal way. Animals will normally be penned individually in metabolism crates to enable the complete collection of excreta passively, and the separation of urine and faeces passively in the case of the goat. The desired goal is to characterise 90% of the total radioactive residue i.e. characterise the destination of

90% of the dosed material. Animals will remain within sight of others. Animals will usually be killed at the designated establishment by a Schedule 1 method. The time of killing needs to be justified to the regulator. For this reason, blood samples will usually need to be taken before and at various intervals immediately after the first dose. This is in order to calculate the time of maximum concentration in the blood. Animals should not normally be killed before this maximum concentration is reached. If frequent blood samples are required, particularly from birds, indwelling cannulae may be used, these will be inserted by non-surgical means. Tissue specimens (skin, fat, muscle, liver and kidney) will be collected post mortem.

Livestock residue studies (OECD 505)

Livestock residue studies will typically involve administration of pesticide to animals by addition to the feed or by dosing. Dosing of cows will usually be conducted by addition of the pesticide on top of their feed twice daily. However, where pesticide is unpalatable it will need to be administered via a dosing gun, capsule or by gavage. Dosing of hens will usually be once daily via an oral capsule. Animals will be dosed for a minimum of 28 days or until residues plateau in milk or eggs, if they have not done so after 28 days.

Milk and eggs will be collected in the normal way. Animals will normally be penned individually so that individual feed intakes can be measured, but they will remain within sight of others and will have nose- to-nose contact with animals of similar treatment group. Poultry will be kept on perforated / slatted / mesh floors to prevent recycling of the pesticide. Animals will usually be killed by a Schedule 1 method at the designated establishment. Tissue specimens will be collected post mortem, so no invasive sampling is required.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects are expected to be mild in nature and the incidence is expected to be low. Blood sampling of laying hens during metabolism studies will lead to haematoma in approximately 50% of cases. However, our experience is that these heal rapidly with no observable consequences to the birds. All other adverse effects are expected to be infrequent i.e. an incidence of less than 1%. All animals will be euthanased by a humane method at the end of each study.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity of all procedures is expected to be mild (99% of animals are expected to fall into the mild category).

What will happen to animals at the end of this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Livestock residue and metabolism studies are not required for all crop protection chemicals and clients usually make strong representations to the regulator to avoid the need to conduct them. However, once the regulator decides that animal feeding or metabolism studies are necessary they usually specify that they need to be carried out to OECD Guidelines for the Testing of Chemicals, Test No. 505: Residues in Livestock or Test No. 503: Metabolism in Livestock respectively. The use of animals is specified in these guidelines. For each study conducted under this licence we will ensure that, as part of the Ethical Review Process, the client has made representations to the regulator questioning the need to conduct an animal feeding study.

Which non-animal alternatives did you consider for use in this project?

As these studies are for regulatory purposes once the regulator decides that animal feeding or metabolism studies are necessary they must be tested in the animals. There are no non-animal alternatives.

Why were they not suitable?

Studies must be carried out in the animal as required for regulatory purposes

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The minimum number of animals is specified in guidance documents and these numbers will be used unless there is a strong case to use more. If more than the minimum recommended number is requested, this will need to be justified by the sponsor and/or regulator and statistical advice will also be taken. In all cases, any increase over the minimum recommended numbers will need to be approved through the AWERB.
Over the five year life of the project approximately 50 studies are likely to be conducted, each providing information to allow different pesticides to be introduced onto the market in a manner which is safe to consumers, animals and the environment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals per study is rigidly laid out in the OECD guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The number of animals per study is rigidly laid out in the OECD guidelines. However the data collected will often be applied for other species. For example data on residues in milk from dairy cows will usually equally apply to dairy goats. In most cases the results of a cattle feeding study will be used to establish animal commodity maximum residue limits for goats, pigs, sheep and horses. Within the poultry group, data on chickens will usually be accepted in lieu of data on other livestock poultry e.g. turkeys, geese and ducks.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The choice of animal, model and method is rigidly laid out in the OECD guidelines, OECD 503 for metabolism studies and OECD 505 for feeding studies:

For metabolism studies, OECD 503 indicates that separate metabolism studies should be conducted in ruminants and poultry. The species of choice are lactating dairy goats and egg-laying hens.

For residue studies, OECD 505 indicates that separate feeding studies should be conducted for ruminants and poultry whenever residues are likely to occur in the feeds of these classes of livestock. The species of choice are lactating dairy cows and egg-laying hens. Data on residues in milk from dairy cows will usually equally apply to dairy goats. In most cases the results of a cattle feeding study will be used to establish animal commodity maximum residue limits for goats, pigs, sheep and horses. Within the poultry group, data on chickens will usually be accepted in lieu of data on other livestock poultry e.g. turkeys, geese and ducks.

It is unlikely that any toxicological effects will result from administration of pesticides at the levels to be used in such studies. In all studies conducted under the previous project licences no adverse effects from the pesticides were observed. It is likely that the sponsor will already have conducted some livestock studies, results from these will inform the AWERB on the likelihood of adverse effects being observed in the residue study. However, twice daily observations of the animals for adverse effects which may be associated with the pesticide will be conducted.

Why can’t you use animals that are less sentient?

The choice of animal, model and method is rigidly laid out in the OECD guidelines, OECD 503 for metabolism studies and OECD 505 for feeding studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Twice daily observations of the animals for adverse effects which may be associated with the pesticide will be conducted.

Animals will usually be acclimatised in their individual pens for a minimum of one week, usually two, before dosing / feeding of the pesticide commences. Individually penned animals will be located within sight of each other to minimise any distress caused by individual penning. For metabolism studies involving one goat, a companion animal will be located within sight of the study goat. Goats will be acclimatised to new diet in group pen before placing in metabolism pen to acclimatise to new pen.

For residue studies administration of the pesticide on top of the daily compound feed allowance for dairy cows has been successfully implemented on most occasions. This refinement means that cows do not have to be restrained for administration of a bolus twice a day for 28 days. However due to the composition and palatability of some products this is not always possible in cattle. When not possible staff will be fully trained in methods of restraint, the animals head will be held securely and lubricant such as liquid paraffin may be used on the dosing gun. This refinement was trialled for laying hens but failed due to palatability problems. This refinement is not possible for metabolism studies as the method of administration is prescribed in OECD 503.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

OECD guidelines, OECD 503: OECD Guidelines for the testing of chemicals, Metabolism in Livestock and OECD 505: OECD Guidelines for the testing of chemicals, Residues in Livestock (sometimes referred to as feeding studies or transfer studies)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attend HOLTIF meetings, be on email lists of ASRU, HOLTIF, NC3RS, Large Animal Research Network and IAT. Discuss 3Rs regularly in AWERB and with NVSs and NACWOS.

132. Endothelial function and vascular homeostasis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cardiovascular disease, Endothelial cells, Vascular Function, Signalling Pathways, Therapeutic Targets

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Endothelial cells form the inner layer lining every blood vessel in the body of vertebrates. The function of endothelial cells is essential to regulate cardiovascular function, including blood pressure, blood clotting and inflammation. Thus, investigating the mechanism regulating and maintaining endothelial function in the adult organism will shed light on the pathological mechanisms driving the onset and progression of cardiovascular diseases including hypertension and 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.

Why is it important to undertake this work?

Around 7.6 million people live with heart and circulatory diseases in the UK with 168,000 annual death from heart and circulatory disease (BHF Stats, https://www.bhf.org.uk/). Although lipid-lowering drugs such as statins have significantly reduced mortality from Cardiovascular Diseases (~25%), significant levels of mortality remain. The work described in this project licence will provide information and increase our knowledge on the mechanisms promoting endothelial and vascular function and shed light on the

pathogenic mechanisms and signalling pathways involved in the onset and progressions of cardiovascular diseases such as hypertension and atherosclerosis. This knowledge will contribute to identifying new therapeutic targets and to inform translational research that aims to improve the condition of patients affected by vascular diseases. The project will employ genetically-modified mice and pharmacological tools which will allow to modulate specific signalling pathways in endothelial cells and the vasculature and to investigate their role in endothelial and vascular homeostasis and cardiovascular diseases. While pharmacological tools such as inhibitors and activators could modulate specific signalling pathways in all tissues and organs, genetic modification permits selective tissue or cell-type targeting of specific pathways.

What outputs do you think you will see at the end of this project?

The project will identify new pathways and mechanisms involved in endothelial and vascular function. The data produced during this project will be included in publications in high-impact international scientific journals and shared with the scientific community at national and international scientific conferences. Also, the data generated will be included in expert peer-reviewed grant, seeking further funding to investigate how the molecular mechanisms and signalling pathways regulating endothelial function control the function of the cardiovascular system in physiological conditions and how their dysregulation promotes the onset and progression of cardiovascular diseases.

Who or what will benefit from these outputs, and how?

The knowledge produced during this project will contribute to advance the vascular research field by expanding our understanding of the signalling and molecular mechanisms involved in the pathophysiology of vascular homeostasis. Thus, the project will give insight into the pathogenic mechanisms associated with cardiovascular disease, with the ultimate goal of identifying novel therapeutics to treat this disorder. As the project has the potential to identify novel therapeutic targets and medicines or biomarkers for vascular diseases, data from this project will contribute to improve current treatments for cardiovascular diseases and will benefit patients suffering from these conditions in the long-term.

How will you look to maximise the outputs of this work?

Findings from this project will be included in publications in peer-reviewed high-impact international scientific journals and will be shared with the scientific community at international scientific meetings. We will take part in initiatives of public engagements through initiatives organised by the British Heart Foundation such as events organised in the BHF charity shops in which researchers in the vascular field explain to the general public the importance of cardiovascular research and how this contributes to saving lives.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To investigate the mechanisms and the signalling pathways regulating endothelial and vascular homeostasis and how their deregulation leads to cardiovascular diseases, we will use an array of in vitro techniques and cell culture. While these in vitro techniques will provide essential data to identify signalling pathways involved in endothelial and vascular homeostasis they lack the biological complexity of an organism and provide limited physiological and pathological insight. Thus, key findings obtained in vitro will be translated in vivo using mice, to reach meaningful results and corroborate our findings. We elected to use mice for our project because mice are the most extensively used animal model for cardiovascular research and genetically modified mouse models allow for rapid establishment of proof-of-principle, which can later be extended into larger animal models and, eventually, into humans.

Typically, what will be done to an animal used in your project?

To perform cardiovascular phenotype studies the animals will be treated with specific inhibitors/activators of pathways we suspect to be involved in the maintenance of normal physiology, or with drugs that activate/inactivate gene expression in genetically modified mice. Typically mice will be pre-treated with established or novel drugs or control substances/vehicles by one route such as oral (i.e. gavage or ingestion with food/water), injections (i.e. subcutaneous, intraperitoneal). In addition, some animals may be subjected to dietary intervention and will receive standard or modified, enriched or deficient diets (altered in fat and/or protein and/or carbohydrate and/or specific drug content) during a variable period. In some animals, we will monitor blood pressure using the non-invasive tail-cuff method. We will collect cells/tissue/organs from terminally anaesthetised animals. Typically animals will be injected at 4-6 weeks of age, and kept for further 20 weeks, depending on the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Treatment with drugs is not expected to cause adverse effects because doses will be selected which are believed to be safe. Animal subjected to treatments will be observed and monitored for any of the following signs: pain, laboured breathing, piloerection, orbital tightening, not eating/ drinking, or loss of body weight. No treatments in this license are anticipated to cause more than minor and transient discomfort. Any animal showing signs of suffering that are greater than minor and transient or in any way compromises normal behaviour will be immediately 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 animal type)?

The overall severity for animals undergoing the above-mentioned procedure is moderate

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because of the dynamic interactions between different cell types forming the vasculature and the interaction between the endothelium and the immune system that typify the maintenance of cardiovascular function and pathological processes occurring in vascular disease, it is necessary to use animals and animal models of cardiovascular disease. Mice are phylogenetically related to humans, with the two species sharing virtually the same set of genes (99% of human genes have direct murine orthologs) and physiology. The cardiac developmental stages observed in mouse and human embryos are comparable, as well as the overall function and physiology of the cardiovascular system, although differences remain mainly due to the difference between the two species in the length of the gestation period, size and lifespan. Because of their genetic and physiological similarities, several mouse models of cardiovascular diseases such as atherosclerosis, hypertension, heart failure mimicking the related human conditions have been established and represent an invaluable tool to understand these diseases. Whilst there are few animal models that faithfully reproduce all the pathology of the analogous human cardiovascular condition, it is possible to produce certain fundamental processes in mice and in genetically modified mice that may either reveal new mechanisms or potential therapeutic targets. Thus, provided one is aware of the limitations of animal models, they play a valuable role in increasing our understanding of the pathophysiology of vascular disease.

Which non-animal alternatives did you consider for use in this project?

We will perform experiments using animals only after having performed and obtained meaningful data on the pathways and mechanisms involved in endothelial and vascular function using cell culture in vitro models. For these experiments, we will use commercially available human endothelial cells in which we will modulate specific signalling pathways using molecular (i.e. small interfering RNA) or pharmacological approaches (i.e small-molecule inhibitors, drugs). We will use these cells to perform traditional biochemical analysis, transcriptomic and proteomic analysis profiling assays. We will also use devices to mimic in vitro the physiological conditions of blood vessels in which endothelial cells are exposed to the forces applied by the flow of blood. In these experimental conditions, we will investigate how modulation of these pathways regulate the communication between different cell types (i.e. smooth muscle cells, immune cells, etc.) by performing co-culture in vitro experiments. We will also take advantage of clinical and genomic data from patients affected by cardiovascular diseases to investigate whether genetic variants associate with cardiovascular disease. Then will study the biological effect of these variants in vitro in using human cells. Together, these in vitro experiments will replace the use of animals in the initial phase of this project to identify signalling pathways and mechanisms regulating the biological processes addressed by this project. Data obtained with these in vitro approaches will then be used to guide a very targeted experimentation of these pathways and mechanisms in the in vivo setting (i.e. whole animal) or ex-vivo (i.e. organs and tissues).

Why were they not suitable?

The studies using animals described in this project form part of a larger initiative that involves the use of cell culture and other in vitro techniques. In vitro systems such as cell culture models are powerful tools to determine molecular mechanisms and investigate signalling pathways in specific cell types that could be relevant to regulate biological processes. However in vitro experiments lack the complexity of a whole organism and do not account for physiological interactions between organs and tissues, different types of cells and the effect of changes in vascular function on tissues and organs physiology and on an overall organism. The onset and progression of cardiovascular diseases such as atherosclerosis are multifactorial and characterised by local (i.e. formation of atherosclerotic plaques in specific area of the aorta and coronary arteries,) and systemic changes (i.e. lipid metabolism, inflammation, increased immune response) which are responsible for the initiation and maintenance of cardiovascular inflammation. Thus, while the in vitro approaches allow to identify potential molecular mechanisms and signalling pathways that regulate vascular function, the in vivo approaches allow us to study their relevance in physiological and pathological contests and to evaluate potential biomarkers and therapeutic targets to treat vascular disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The employment of commercially available human cells for the in vitro experiments together with the use of devices to mimic in vitro the physiological conditions of blood flow on endothelial cells, and the availability of clinical data from patients with cardiovascular diseases, will allow to reduce the number of animals and to design a very targeted animal modelling approach. In addition, our extensive experience in pre-clinical models entails we have a substantial bank of data upon which to draw to establish the minimum number of animals required to detect a statistically significant effect in the experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used statistical methods to determine the smallest number of animals to use in each set of experiments. We used NC3Rs' experimental design assistant which helps with the experimental design and statistical analysis plans by providing support for randomisation and blinding, sample size calculations and advices on the more appropriate statistical analysis methods for each set of experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Minimum numbers of breeding pairs will be mated to provide offspring to enable ex vivo and in vivo experimentation. Working on the basis that a breeding pair produces a litter once a month of approximately 8 animals we would need approximately 4-6 breeding pairs to provide a minimum of 1 animal per day for experimentation. Our breeding design will allow to produce perfectly stage- and strain-matched controls animals. Animal of both

sexes will be used in the experiments. The members of the group will share tissues harvested from these animals to analyse different aspects of the project, thus maximising the use of experimental animals and avoid 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All of the models described in this licence are mild to moderate; there are no models that fall into the severe category. Each is well-validated and widely used for investigation of the mechanisms underpinning cardiovascular diseases, and gleans robust & informative with minimal animal suffering.

Why can’t you use animals that are less sentient?

The work will be conducted exclusively in mice to enable the exploitation of transgenic technology that enables gene targeting (i.e. gene deletion or gene expression) in the whole animal or in a tissue- specific manner. Relevant to this project, the availability of mouse mutants in which gene deletion can be pharmacologically induced in the endothelium makes the use of these animals an asset to determine the role of specific genes in the function of the endothelium, the vascular system and cardiovascular diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All protocols described in this PPL involves minimally invasive techniques and no animals will undergo invasive procedures such as surgeries. For instance, we will use non-invasive ultrasound imaging systems and/or cuff systems to measure blood pressure. Analgesics and anaesthetics will be used whenever necessary, and close monitoring of all animals undergoing licensed procedures will be undertaken. Any animal deemed to be experiencing unnecessary suffering by the investigator or veterinarian will be culled immediately by an approved method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will monitor published scientific literature in which similar models described in this licence have been used, to ensure that we will conduct the experiments in the most refined way. We will check and follow the latest guidelines on animal welfare. All researchers involved in the current project will follow the guidelines. “The Responsibility in the use of animals in bioscience research” general guidance document will be used as a reference document, as it sets out the expectations of the funding bodies for the use of animals in research. The updated ARRIVE guidelines (2.0), designed for transparent reporting of

animal research methods and findings, will be consulted for the planning and design of new experiments, together with the “Guidelines for planning and conducting high-quality research and testing on animals” (ref. 1) and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (ref. 2). Finally, we will follow the guidelines of the UK Research Integrity Office (UKRIO) and will aim to register our study protocols in an international online register of protocols for preclinical animal studies.

References:

Smith AJ (2020) Guidelines for planning and conducting high-quality research and testing on animals Lab. Anim. Res. 36:21. doi: 10.1186/s42826-020-00054-0. eCollection 2020.

Smith AJ et al. (2018) PREPARE: guidelines for planning animal research and testing. Lab. Anim. 52(2):135-141. doi: 10.1177/0023677217724823.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

PIL holders working under this licence will regularly attend NC3R, BPS or similar meetings to keep abreast of new developments, and to maintain awareness of current best practices.

133. The Regulation of Energy Balance

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

Body composition, Calorie restriction, Energy Expenditure, Healthspan

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the project is to investigate the complex mechanisms regulating body weight which ultimately affects health and lifespan.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With a better understanding of the mechanisms controlling body weight we can help tackle 2 key worldwide issues – obesity and an increasing ageing population.

What outputs do you think you will see at the end of this project?

Results will be disseminated by peer reviewed publications. Outputs will further our understanding of the regulation of body composition remodelling under negative (calorie

restriction) or positive (high fat diet) energy balance in males and females. The impact on reproductive investment under calorie restriction will be determined.

Who or what will benefit from these outputs, and how?

Outputs from the work of this licence are specifically related to understanding the fundamental biology of energy balance controlling our body weight. Our research outputs are clearly relevant to major public health issues such as obesity and ageing. Calorie restriction, ie. dieting is the primary General Practitioner and self-prescribed treatment against obesity. In many species calorie restriction improves health and extends lifespan with recent work finding similar benefits in humans. Establishing the mechanisms by which calorie restriction exerts its beneficial effects is a key priority for research because it may open the door to interventions that will allow us to capture the health benefits of eating less and ultimately retard the rate of ageing. Benefits will be realised by other scientists working in the field of ageing as well as healthcare, specifically the quality of life for the elderly and improvements in health of obese population.

How will you look to maximise the outputs of this work?

We are committed to open science and generated data will be deposited in our open access database. We have a strong history of promoting our work among the academic and lay communities ensuring the results are widely available. Our work will be published in high impact journals with summary forms available via social media, ResearchGate, academia.edu etc. We have a strong network of collaborators worldwide who have contributed in various ways to our research. Tissues are regularly shared with our collaborators generate maximum output from minimal numbers of animals.

Species and numbers of animals expected to be used

- Mice: 1150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our work will use mice, wild type and genetically altered (GA). The mouse is an ideal choice because they share a mammalian physiology and high genetic homology with humans. There are of course also differences between mice and humans that we should not ignore, but as a first step, studies of mice allow us to carry out calorie restriction studies and explore ideas about ageing that can then be expanded in other models and humans themselves.

The majority of work on this licence will use the C57BL/6 mouse which is a well-established model for the study of energy balance. The genome of the C57BL/6 mouse has been fully sequenced and therefore this model is indispensable for our studies.

The use of GA mouse models will allow us to validate the function of any gene of interest we identify as playing a key role in energy balance.

Generally, our calorie restriction protocol is introduced at 20 weeks of age, approximately equivalent to early human adulthood, and close to the time when mice reach skeletal maturity. Allowing mice to mature avoids impacts of calorie restriction on developmental processes. In the case of GA animals which may become obese rapidly an earlier start age may be necessary for start of protocol. The duration of our studies is generally 3 months but be shorter or longer.

Typically, what will be done to an animal used in your project?

A typical calorie restriction study would involve 6 procedures generally performed pre- and post- dietary regime (ie. 12 weeks apart) or following the administration of substances:

Single housing,

Intraperitoneal transmitter implantation for logging body temperature and activity continuously throughout the study.

Dual x-ray absorptiometry (DXA) or Echo MRI. Scanning may be carried out at any stage and on multiple occasions throughout the protocol. For DXA, scans are generally every 4 weeks but no more than 10 times over a 3-month period. Echo MRI may be weekly.

Glucose tolerance tests (GTT) Mice are fasted, injected with glucose and blood withdrawn by tail prick. Tests take 2 hours from injection.

Resting metabolic rate (RMR) is a non-regulated procedure.

Calorie restriction by reducing energy provided to mice by a % of their individual ad libitum intake Where appropriate alternative Energy Expenditure methods may be used:

Doubly labelled water technique requires intraperitoneal injection followed by 2 tail blood samples taken between 1 and 48hrs post-injection.

TSE Phenomaster

Daily energy expenditure may be measured using home cage analysis systems. Measurements are made over a 72-hr period (includes a 24-hr acclimatisation) at the end of the study.

Animals may also be Pair-fed or have their dietary content (protein, fat, carbohydrates) manipulated

Withdrawal of blood Repeated blood samples may be taken through the course of the study. Volumes and frequencies will comply with the local LASA guidelines unless otherwise stated.

Non-central administration of substances and relevant control. Several routes (topical application, injection via the subcutaneous, intraperitoneal, and oral routes, such as gavage) may be used over varying durations.

What are the expected impacts and/or adverse effects for the animals during your project?

Anticipated adverse effects are reported below.

Stress Single housing may cause stress. When transmitters are used, they will be single housed from time of surgery ~12wk for 20 weeks

Weight loss is expected to occur in calorie/ dietary restriction and pair feeding studies. Calorie restriction is known to improve health & lifespan and weight loss is not detrimental unless excessive. Animals used in dietary manipulation studies may also lose weight. Following DXA which uses gas anaesthesia, in a few cases, a small drop in body weight (and food intake) may occur (generally no more than 1g of either) may occur. This is generally overnight, and mice recover within 24 hours.

Torpor is defined as a body temperature <31°C. This is a natural energy saving mechanism and not detrimental. Calorie restricted or pair-fed mice are expected to experience torpor. Some GA models, e.g. ob/ob and db/db also experience spontaneous torpor due to the lack of functioning leptin system.

Hypothermia is a drop in body temperature which some GA models, e.g. ob/ob and db/db spontaneously experience. The duration should not be greater than 8 hours.

Food Anticipatory Activity known as FAA is a marked increase in activity in the 2-3 hours prior to food provision. FAA is thought to be normal food seeking behaviour.

Risk of infection or changes in behaviour following surgery. There is also a slight risk of wound re- opening.

Hypo/ hyperglycemia may occur in rare instances immediately post injection of glucose for GTT.

Excessive urination in ob/ob and db/db mice which become diabetic. These may lead to further prepuce disorders.

Skin conditions Some mice may develop dry patches of skin which may become irritated, inflamed (red). We have found this in the ob/ob and db/db mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

75% of mice are expected to suffer moderate adverse effects due to the effects of transmitter implantation surgery. The rest of the mice should suffer no more than mild adverse effects.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This work requires a whole animal system. There are no feasible alternatives which allow us to meet our objective. With close genetic and physiological similarities to humans, the mouse is the premier mammalian model system for genetic research. Using mice, we can probe the complex physiological systems that humans and mammals share, ie. immune, hormonal, nervous, cardiovascular, skeletal etc. With the key objective of this license to investigate energy balance, the mouse is ideal, specifically the C57BL/6 mouse which is a well-established model for such studies and is known to respond positively to calorie restriction. We have an extensive data set from the C57BL/6 which we have used for the past 12 years to characterise the response to calorie restriction. Measures range from physiology to genes, hormones to behaviour, body temperature to metabolomics etc. Using mouse models we can manipulate intricate interactions of biology, behaviour and environment which would be impossible using in vitro modelling techniques which lack the whole body integrated responses of different cell types, hormones etc. From our post-mortem we collect 22 tissues. From the dissection weights, tissue remodelling following the dietary interventions can be determined. All tissues are stored appropriately at -80°C for further analysis eg. transcriptomics allowing us to pin-point tissue and gene specific responses. Most importantly we have access to the hypothalamic region of the brain which controls energy balance regulation. GA mouse models are also invaluable to our research determining the function of genes involved in energy balance.

Which non-animal alternatives did you consider for use in this project?

There is no feasible alternative to investigation of the whole body regulation of energy balance. Calorie restriction for instance elicits varying response in numerous tissues, some losing and some gaining mass at different timepoints and levels.

If appropriate we will use tissues available from specific data banks such as the NIA https://www.nia.nih.gov/research/dab/aged-rodent-tissue-bank-handbook/available-tissues

Why were they not suitable?

Energy balance is a complex interaction of many factors. While cell culture work may test responses to compounds how this response is integrated in a whole body system cannot 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?

All experiments performed will be based on the principle of using the least number of animals compatible with having sufficient statistical power to achieve statistical significance using appropriate statistical tests.

Our calorie restriction protocol using a graded approach at 4 levels from 10 to 40% less food, plus control group, with an n of 8-10 per group for a short-term study this totals 50 mice. We will use both sexes and several GA mice. (250)

Leptin replacement study = 50
Breeding may be required for some GA models (allow for 250) BBSRC Grant submission 2022 = 500
Possible pilot studies = 100

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Study plans are rigorously designed to obtain the maximum information from the minimum number of animals. As part of the design, we seek statistical advice to ensure animal numbers are adequate to meet statistical significance.

We have used the graded calorie restriction protocol for 12 years. Our protocol is robust and generates reproducible data. Recent experience with GA animals indicated starting with higher numbers to address unanticipated responses to surgery.

We have a very stringent dissection procedure taking 22 organs for further analysis. This ensures the data from every animal is utilised to its full potential and avoids repetition of studies. We have a large collaboration network and have provided several tissues to other research groups with an expertise in specific fields.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where available GA animals will be bought in from external suppliers. Where appropriate breeding of GA models will be carried out in-house under the guidance of staff to achieve the most efficient breeding protocol.

As stated above we collect 22 different tissues for further analysis. Tissues are stored at -80°C for future analysis. We regularly share tissues, reducing repetition of studies and unnecessary use of animals.

Where appropriate pilot studies will be used to determine efficacy of administered substances or use of GA mice. A successful pilot study will also help to guide refinements and humane endpoints.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

C57BL/6 mice are the most refined model for use in calorie restriction. Where appropriate other models may be used.

Calorie restriction will be used on models with ‘normal’ food intake. Pair-feeding is used when we know animals are hyperphagic, ie eating excessive amounts. Pair-feeding will reduce their food intake to that of appropriate controls.

Where GA animals are used relevant approval will be requested and adverse effects assessed.

While implanted transmitters to measure physical activity and body temperature involve an initial surgery they provide non-invasive continuous assessment of the parameters. In addition, the transmitters are beneficial post-surgery allowing us to monitor recovery of the mice via their body temperature.

Why can’t you use animals that are less sentient?

Our calorie restriction protocol is initiated at 20 weeks, equivalent to early adulthood in humans. Beginning earlier has been shown to have detrimental effects on development.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All appropriate refinement measures will be applied to eliminate or minimise welfare costs ie pain, suffering, distress or lasting harm experienced by animals during a study.

Advances in 3Rs are received via the NC3Rs newsletter. The most refined methods are currently used and updated where appropriate following discussion with the NACWOs/ NVS, eg cupping/ tube handling has recently been introduced for all animal handling to reduce stress.

Refinements arising during a study will be applied to future experiments.

Monitoring methods were refined to use body condition score as opposed to body weight loss.

Equipment and methods will be continuously reviewed and updated should they more refined alternatives become available in the future.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies

ARRIVE: Animal Research Reporting In Vivo Experiments, 2020
https://www.nc3rs.org.uk/news/new- arrive-guidelines-2-0-released

The PREPARE guidelines, https://norecopa.no/prepare

RSPCA and LASA, 2015, Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies. A report by the RSPCA Research Animals Department and LASA Education, Training and Ethics Section. (M. Jennings ed.) Current Publications | LASA

Wilkinson, MJA, et al, Lab Animals 2020; vol 54(3); 225-238; Progressing the care, husbandry and management of ageing mice used in scientific studies https://journals.sagepub.com/doi/10.1177/0023677219865291

Pettan-Brewer, C. and M. Treuting, P., 2011. Practical pathology of aging mice. Pathobiology of Aging & Age-related Diseases, 1(1), p.7202. https://pubmed.ncbi.nlm.nih.gov/22953032/

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 stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I subscribe to NC3R and receive regular emails, from NC3R News which provides updated information.

134. Teleost models of haematopoietic 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

Bone Marrow Failure, Leukaemia, zebrafish, haematopoiesis, therapy

Animal types	Life stages
Zebra fish (Danio rerio)	adult, juvenile, embryo, neonate
Killifish	juvenile, adult, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of our project is to better understand the development of blood cancers and other related blood conditions. Our studies address the genetic basis of these diseases and how they evolve. We will also use models of these diseases to define new 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?

We have a large amount of knowledge about errors (known as mutations) in genes that lead to the development of blood diseases (bone marrow failure syndromes such as Diamond-Blackfan Anaemia) and blood cancer (leukaemias). Much less is known about the consequences of these mutations, how they result in disease, and what modifies how severe the disease is.
Some blood cancer causing mutations can also be found in people without clear evidence of disease. These individuals have an increased risk of blood cancers. This risk increases

as we get older. What is lacking is a better understanding of what converts mutations in one gene into a cancer. This can be because other mutations have been acquired as part of the aging process. Alternatively it can be because the environment in which the cells with the mutation sit is abnormal. There may be other additional factors contributing to this process, such as how the cell gets its energy (metabolism) or how much of a gene is present or other ways that we don't know yet. These non-genetic ways such as these that occur due old age are called epigenetic effects.

Our project aims to understand the process of the development of blood cancers and related blood diseases. In particular we are looking to find out how age contributes to the development of blood cancers using a type of fish that ages very rapidly. We are also looking to find new treatments for these diseases. This includes identifying therapies that might potentially prevent the onset of cancer. The ultimate goal is to provide a detailed understanding of how blood diseases evolve to facilitate new treatments that improve human health.

What outputs do you think you will see at the end of this project?

This project will generate new information about what causes the development of blood cancers and related diseases. Our project also aims to define new treatments for blood cancers. The outputs will include datasets (genetic sequencing data) that will be made publicly available and scientific publications presented at scientific meetings and published in high impact journals and potentially new drugs that can be moved to the next steps of testing in human samples and eventually patients.

Who or what will benefit from these outputs, and how?

By the end of the project we aim to have detailed information on

How leukaemia evolves from the combination of two or more genetic mutations in combination

Why people with inherited predisposition to leukaemia go on to get leukaemia later in life

Potential new drug treatments that can target leukaemia cells which carry specific genetic mutations.

How different energy sources are used in models of Diamond-Blackfan Anaemia and how we may utilise this knowledge to create novel treatments

These benefits will be across the scientific community and clinical researchers in the leukaemia, anaemia and bone marrow failure field.

How will you look to maximise the outputs of this work?

I will publish the results arising from the studies in this licence in the scientific literature. This will be maximised by presenting the work at national and international conferences, sharing data, publishing on pre-print servers (e.g BioRixv - this means work can be published prior to peer review in a timely manner) . I also have strong links with patient support groups (MDS UK, DBA UK and Children with Cancer) as well as Cancer Research UK, with whom I regularly engage with to disseminate the results of my research across patient groups and their families.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 13700
- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Zebrafish are the least sentient animal that develops blood akin to that seen in humans. The programming of blood cell development occurs in a similar manner to that seen in humans. In addition, using fluorescently labelled blood cells, we can observe the development of blood cells and we can see the development of blood and blood cancers in real time using a microscope, especially during the early stages of development (first 5 days) when the embryos are transparent. We think that for conditions where there is an inherited predisposition to blood cancers, how blood is made during development contributes to the development of leukaemia or bone marrow failure later in life.

Therefore zebrafish are the best model to use to study this process as it can be studied in the embryos which are transparent and develop outside the mother.For our studies of blood cancers that are acquired in older adults, we know there is an increase in the incidence of these cancers as we age. We use killifish to model these cancers rather than zebrafish because they naturally age very quickly over only a few months compared to 2-3 years with zebrafish, and show the signs of aging we observe in humans. Using killifish allows us to determine the effects of aging in combination with genetic errors over time without having to wait many years to obtain results.

The use of both of these types of fish is complementary in our approaches to minimise harm (using zebrafish for mainly developmental studies before 5 days of life where possible and killifish where we would like to assess specific effects of aging).

Typically, what will be done to an animal used in your project?

Adult zebrafish and killifish will be bred to maintain stocks required and to generate embryos/larvae for experiments where necessary. These fish will live a normal, healthy life as any aquarium fish. A small biopsy of the tail fin will be taken from some fish in order to determine certain genetic characteristics, typically only once in an animal's lifetime.

We will use fish with fluorescently labelled blood cells to aid our assessment of blood development. This will allow us to visualise blood cancer development while the fish is alive by using a microscope. Fish will be anaesthetised to allow this assessment which may be done several times (up to 8, however usually once or twice) to observe the effects over time. We may also take a small sample of blood from the fish to look at it in more detail.

We also aim to look for possible new treatments. This means that some fish will be treated with compounds aimed to prevent or treat the blood diseases we are studying. The majority of this work will be undertaken prior to the onset of independent feeding. An adult fish receiving a treatment would only receive one treatment over its lifespan. The

treatments are usually administered in the fishes water, although sometimes we may need to administer the treatments directly into the fishes digestive system, which is done when they are asleep using a small tube.

The forms of leukaemia we see as a result of older age are thought to be partly caused by the environment in which the leukaemia cells grow. For a small subset of animals (around 1%) we will take blood stem cells from one animal after euthanasia, and transplant them into another, using a small injection into the belly of the fish. This will help us understand whether if we change the "aging" environment we can prevent leukaemia. This will only be undertaken once in a fishes lifetime.

Most blood diseases occur because of the presence of genetic abnormalities. In humans some of these genetic abnormalities are inherited. In order to study these diseases accurately we will introduce these same genetic abnormalities into the fish. This is typically done when the fish are at the egg stage so that all the cells of the fish carry the genetic defect. This is done using microinjection of gene editing RNA (CRISPRs) at the one-cell stage. However, in some instances (around 5%) we will induce these changes in adults and in only some of the tissues in the fish (usually blood for our studies). This is done using drugs such as tamoxifen or heat to activate the genetic changes. This is important most human cancers evolve due to several genetic changes that occur only within the tissue of that cancer as we age.

What are the expected impacts and/or adverse effects for the animals during your project?

For the majority of fish, adverse effects are not expected. We know this because we have experience with many of these models already from our prior work. For protocols where there are treatments to the fish, or where the fish develop the diseases we are studying (anaemia or leukaemia) there may be adverse effects in some animals. The expected adverse effects may include reduced food intake, reduced or abnormal swimming, infection and some developmental abnormalities such as small size or heart problems. This will affect a very small number of animals for a maximum of a few days where the animals are undergoing experimental purposes within the limits of the designated protocols.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity limit for the majority of experiments is mild. Some of our animals may develop features of the diseases we are studying such as leukaemia. We will only permit this to happen if it is essential for our protocol, for example testing the effects of a new drug to treat leukaemia. In such cases we don't know what the effects of the drug on the fish in this setting might be, so a small number of animals may endure moderate severity limit in these experiments. Procedures are in place to closely monitor these effects during these protocols. These include assessing animals changes in swimming behaviour, pallor, or reduced growth.

We know that our zebrafish model of Diamond-Blackfan anaemia has some features of this condition. Most of these are obvious during very early development prior to the onset of independent feeding. We only raise animals that are healthy prior to the onset of

independent feeding. These animals show signs of being small but otherwise appear indistinguishable from their siblings during normal aquarium lifespan. Although we do not observe any clear signs of harm to the vast majority of these animals we attribe a mild phenotype to these animals since we may not be able to detect this. The proportion of animals in a moderate severity category we expect to be less than 1% based on our prior experience.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Blood cancers develop as a result of genetic defects. While these can be and are studied using cells in a dish or cells directly from patients there are 2 main confounding factors that mean animals are needed for our studies:

We are interested in how blood cancers develop over time. It is not feasible to follow humans over many decades and examine their blood system in depth regularly to assess the effects of different genetic errors in their blood. The fish species used in this project (particularly killifish) age very rapidly with features of aging that we see in humans. This allows us to see the effects of a genetic error over a lifespan.

The effects of genetic errors cannot be assessed in isolation outside their natural environment. We are particularly interested in myelodysplastic syndrome (MDS) which is a kind of pre-leukaemia. There is good evidence that chronic inflammation, which comes from cells that are in the vicinity of blood, but not the actual blood cells themselves contribute to the development of this disease. Therefore looking at cells in a dish or even from a patient outside the body does not give us the necessary understanding about what happens to the cells in their natural environment and how this contributes to leukaemia.

Which non-animal alternatives did you consider for use in this project?

We have other projects in our lab that are being conducted in parallel using human tissue from MDS patients and normal humans. Our studies using fish are designed to answer the questions it is not possible to address using human samples. Importantly, we will use human tissue to validate our findings from fish work. These combined studies will help us in addressing the important next questions so we only use animals when they are absolutely needed.

We also considered using 3D bone marrow scaffolds to grow human stem cells. However, disease cells do not grow well in these and we cannot readily test the environment in which the cells grow using this technique because disease cells do not exist in isolation but are supported by lots of other types of cells such as bone cells and blood vessel lining cells.

Why were they not suitable?

Bone marrow samples from patients and normal individuals provide critical information about the biology of blood development and disease. However these only give a snapshot of information at the point in time when the samples are taken. As these are invasive tests for patients it is not ethical to do sequential tests, rather we use surplus tissue following diagnostic testing for our analysis. Our studies in fish permit us to assess what happens to blood over time due to certain genetic abnormalities and how this leads to leukaemia.

Bone marrow scaffolds allow short term culture of human blood stem cells. To date however these scaffolds still rely on animal material (bovine bones) or do not provide extended longer term support for cell growth and so again do not allow us to assess over time. Furthermore, these changes occur over years to decades in humans, and so to study effects over a lifespan with natural aging there is no viable alternative than using a rapidly aging organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals we will use is based on our usage of animals in prior project licences and the proposed experiments we will undertake in this new licence. Our approach of using as many animals under the age 5dpf for the majority of experiments means that we are able to use relatively few juvenile/adult animals for our project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3Rs experimental design assistance to allow us to calculate the minimum number of animals needed for our studies. We have also gained considerable experience from our prior studies. These allow us to more accurately assess the range of what is normal and the kind of measurable changes we expect and to allow us to predict with more certainty the actual number of animals we will require to assess our endpoints. Where possible relevant experiments will be conducted prior to the onset of independent feeding to reduce the use of older fry and adults.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Some of our studies use methods to knock out genes of interest that we think play a role in cancer or might protect from cancer or blood disorders. We have adopted methods that allow us to do this only in embryos before 5 days which in the past would have required developing and breeding a knockout animal for 2-3 generations to obtain the same information. These methods dramatically reduce the need to keep as many fish and allow us to prioritise the genes of interest to undertake the most important and relevant experiments only.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using zebrafish and Killifish in our experiments. We have structured our experimental approach to use animals before the onset of independent feeding where possible. Killifish are used instead of zebrafish for experiments in which we want to assess the effects of aging of cells (pre-leukaemia cells, or the cells of the environment) because killifish age very rapidly and allows us to conduct our experiments in a more timely manner. By contrast zebrafish produce more embryos and have a wider spectrum of existing tools and methods for use in experiments where we are able to use direct imaging and animals before the onset of independent feeding.

Why can’t you use animals that are less sentient?

We are making every effort to balance harm/benefit and to refine our methods and to use fewer animals in accordance with the 3Rs. In particular the majority of our studies will be undertaken in immature forms or terminally anaesthetised animals. These experiments will help guide the experiments we need to undertake in larval and adult forms. For example we will define the most important genetic defects that may protect from cancer in early embryos using a surrogate output, such as the number of blood stem cells present, before assessing if this is the case in adults.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

I have developed structured standard operating procedures to follow for experiments that involve larvae and adults. These stepwise protocols are laminated so they can be utilised in the fish facility in real time for the relevant experiments and provide clear instructions on timings and monitoring of each component of the protocol.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow the PREPARE guidelines (Planning research and experimental procedures on Animals) to ensure experiments adhere to the standards for animal research. I will utilise the ARRIVE (Animal research: Reporting of In Vivo Experiments) guidelines for reporting. Animal lines that are not in current use will be sperm frozen in order to minimise live animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will remain updated using local and national channels that inform me about 3Rs updates. First our local biological services unit provide regular 3Rs updates. Second, our fish facility provide regular advise and updates on 3Rs specific to fish studies. And finally, I subscribe to the monthly updates from the NC3Rs website and their twitter channel. I will use this information to incorporate relevant advances into my work in a timely manner.

135. Understanding the mechanisms mediating local and whole-body functions of the intestine in health and disease.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

stem cells, regeneration, cancer, enteroendocrine system, whole-body health

Animal types	Life stages
Mice	embryo, juvenile, adult, pregnant, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to answer three fundamental questions about gut health and disease: a) How does the gut repair following damage or infection? b) How do intestinal diseases, such as cancer or inflammation arise? c) How does gut illness impact whole-body 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?

The gut performs vital functions, including the absorption and digestion of nutrients and is the body’s defence against ingested harmful substances and infections. Maintaining the capacity to repair itself following injury—a process known as regeneration— is key to

preserving gut integrity and the fitness of the entire body. However, because of its regenerative power, the gut is highly susceptible to cancer and other diseases. Therefore, understanding these fundamental processes is important to develop treatments to aid gut repair and prevent gut disease and its impact to whole-body wellbeing.

What outputs do you think you will see at the end of this project?

Towards the end of this project, we will have a more in depth understanding of the processes that promote repair of the intestine and those that may cause intestinal disease, such as cancer. We will also obtain a more detailed understanding of how and why break down of intestinal function affects the health of multiple organs within the body.

This knowledge will lead to new publications in this field of research and contribute knowledge useful towards the design of treatments for intestinal disease.

Who or what will benefit from these outputs, and how?

The most immediate beneficiaries of this work will be the academic and scientific community interested in the study of stem cells and intestinal biology.

Colorectal cancer (CRC) is the third most deadly type of cancer worldwide. Over 40,000 people per year have been diagnosed with CRC in the UK between 2016 and 2018. During this period, CRC accounted for 11% of all cancer deaths in the UK. An estimated 230,000 people diagnosed with CRC between 1991 and 2010 were alive at the end of 2010.

Inflammatory bowel disease (IBD), causing prolonged inflammation of the digestive tract, affects at least 1 in 250 people in the UK. Patients with IBD are at higher risk of developing CRC. UK prevalence estimates for IBD range from 328 cases per 100,000 people in the 1990s to 970 cases per 100,000 people in 2017.

In the longer term, our work may be of impact to healthcare professionals and pharmaceutical companies interested in the development and application of therapeutic approaches aimed to improve intestinal function and prevent or revert intestinal malignancies, including CRC and IBD.

How will you look to maximise the outputs of this work?

We will maximise the output and impact of our research through several means: 1- Collaboration with basic and clinician scientists.
Communication of research results in scientific meetings/seminars.

Dissemination of our work and its impact to society to the general public through public engagement events.

Publication of results through Fully Open access platforms/journals.

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.

Explain why you are using these types of animals and your choice of life stages.

We will firstly and primarily use the adult fruit fly, also known as *Drosophila melanogaster*, as a model system to perform large scale/unbiased experiments in live animals to assess how intestinal homeostasis, repair and cancer are regulated. However, translation of our work into live mammals will provide greater clinical relevance to our fruit fly data. Mice (*Mus musculus*) are the gold-standard laboratory animal for research on intestinal health and disease. Due to their close physiology and genetic conservation with humans, work in live mouse models has been instrumental for the discovery of processes regulating intestinal health and disease in humans. Therefore, mice represent the most appropriate live mammalian model of research for the translation of our fly work. Because our work is based on the study of the adult intestine and its interaction with surrounding tissues and organs, adult is the life stage we need to in our mouse research and using live animals rather than cultured cells is essential, as available cell culture systems fail to recapitulate the tissues and organs that surround the intestine in real life.

Typically, what will be done to an animal used in your project?

Animal breeding and maintenance.

Whole body irradiation to induce intestinal damage. Single procedure. Total duration of the experiment: 3 days. Or;

c-Feeding of drugs or chemicals to induce intestinal damage, inflammation or cancer initiation, the latter known as tumourigenesis. Intestinal damage: single procedure; Total duration of the experiment: 5-7 days. Inflammation or cancer initiation: at least 3-day procedure. Experiments last between 4 and 180 days.

Injection or feeding of drugs. Single or multiple occasions depending on the process and agent. Total duration of the experiment: 5-7 days to assess the effect on intestinal repair; between 4 and 180 days to assess the effect on cancer initiation.

Injections to: a) induce gene expression (1 to 3 times per experiment); b) incorporate a cell labelling agent (single procedure)

Injections or surgery for introduction of cancer cells. Done as a single procedure. Total duration of the experiment is 180 days.

What are the expected impacts and/or adverse effects for the animals during your project?

Injections might cause mild discomfort/pain. This only lasts for a few minutes, while the procedure is carried out. Pain relief is used where appropriate.
Our intestinal regeneration experiments lead to no visible signs of pain or physical deterioration.

Experiments leading to the generation of cancer can last a maximum of 180 days. Induction of intestinal inflammation and cancer initiation lead to weight loss, possible whole-body discomfort and lethargy.

These are physical signs we closely monitor for, are manifested towards the last few days of the experiment until the animals reach what is considered end point, as per the established project licence guidelines.

Surgery is performed under anaesthesia and appropriate analgesia to avoid 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)?

All our experiments are of mild to moderate severity.

Intestinal regeneration experiments are of mild severity (80-100% of the animals), moderate severity (0- 20% of the animals).

Induction of chronic intestinal inflammation and tumourigenesis are of moderate severity (80-100% of the animals), mild severity (0-20% of the animals).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We firstly and primarily use the adult fruit fly (*Drosophila melanogaster*) in our research, to perform large scale/unbiased experiments in live animals to assess how intestinal equilibrium, repair and cancer are regulated. However, translation of our work into live mammals will provide greater clinical relevance to our fruit fly data. Mice (*Mus musculus*) are the gold-standard laboratory animal for research on intestinal health and disease. Due to their close physiology and genetic conservation with humans, work in live mouse models has been instrumental for the discovery of processes regulating intestinal health and disease. Therefore, mice represent the most appropriate live mammalian model of research for the translation of our fruit fly work.

Which non-animal alternatives did you consider for use in this project?

We firstly and primarily use the adult fruit fly (*Drosophila melanogaster*), a model system whose use does not require regulation by the Home Office and, therefore is not part of this application.

Intestinal tissue grown in the laboratory, commonly known as organoids, is often a good substitute to the use of live animals, which we will considered using to complement some of our fruit fly studies on the intestine. However, organoids are not suitable to study the interaction between the intestine and associated tissues or distant organs.

Why were they not suitable?

While laboratory-grown intestinal tissues are useful to assess processes restricted to intestinal cells themselves, they are less suitable to study how the intestine interacts with its surrounding environment. These types of studies are only possible when the whole organism is considered. Importantly, since the large majority of our work is based on the study of the adult intestine and its interaction with other tissues and organs, the use of live mice rather than laboratory-grown intestinal tissue is essential for the translation of our fly work into mammalian 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?

The techniques we plan to perform using live mouse models are well established and have been previously done by us or collaborators with the highest expertise in mouse models of intestinal regeneration and cancer. Most of our experiments will involve single or double gene combination and, in general, 1 in 2 to 1 in 16 of the mice generated will be used in experiments. Tumourigenesis experiments require a minimum of 10-15 animals and regeneration experiments require a minimum of 6 animals per genotype/condition, to obtain statistical power.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We firstly and primarily use fruit flies— the core research model organism in our laboratory—to identify genes regulating intestinal homeostasis, regeneration and tumourigenesis. We then translate key findings from our fly work into mice. This greatly reduce the number of animals used. As part of our tumourigenesis studies we propose to do transplantations of intestinal cell lines and tissue into recipient mice. This approach will be useful to address interactions between intestinal tumours and surrounding tissues without the need to generate complex genetically modified mice, hence also reducing the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whenever possible, littermates will be used as controls for experiments or to set up new breedings that will provide a higher rate of desired animals. For example, in cases where we want to analyse the effect of gene ‘x’ on the intestine, animals with only one abnormal copy of gene ‘x’ will be used as controls and compared to those animals where the two copies of gene ‘x’ are abnormal. We will try to minimise excess mice by maintaining stock animals carrying as many multiple genetic alterations as possible.

Surplus material generated through ear identification markings will be used for genotyping

purposes. For any genotype and/or treatment where tissue samples already exist, we will use that to test our hypothesis (e.g., assessing gene/protein levels in the context of mouse intestinal regeneration or tumourigenesis) rather than generating new mice. In order to maximise the data obtained per animal, whenever possible, we will use longitudinal studies to assess phenotypes and, when culling an experimental animal, we will collect samples from as many tissues of potential interest as possible and process samples in multiple ways to account for different future experimental needs. For example, we will collect samples of the intestine as frozen tissue, fixed tissue and on RNA preserving solution for future experiments requiring protein extraction, tissue staining or gene expression assessment, respectively.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 adult mice to complement our research on the adult fruit fly *Drosophila melanogaster* intestine. The use of this animal model and age are important to assess the clinical/translational relevance of intestinal regeneration and cancer discoveries made in flies.

Genetic modifications in mice are done in a temporal and tissue specific fashion to avoid any unnecessary impact to organ function and associated health decline caused by broad/non-specific effects of genetic manipulations. In all cases, signs are monitored very carefully by highly expert staff familiar with every model used so suffering can be kept within or below moderate limits or animals are killed.
Why can’t you use animals that are less sentient?

Our research is focused on studying how the adult intestine interacts with its microenvironment and other organs in the body during the process of intestinal regeneration and also in malignant conditions, such as during tumourigenesis and intestinal inflammation. Therefore, we must do our research on live, adult mice, whose body functions similarly to that of humans and can therefore best recapitulate human intestinal health and disease. Having said that, we do most of our research in the less sentient model system, such as the fruit fly. Therefore, we use mice as a complement to our fly work rather than as the main model system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Examples of refinement measures include:

Study animals are weighed, and tumours measured at regular intervals to detect early clinical signs.

Mice will be inspected at least twice daily for the first 2 days following surgery. Wounds will be monitored closely for signs of local infection and mice will be monitored for signs of pain/discomfort. Aseptic techniques will be used during surgery and analgesia is given pre- and post- surgery as advised by the NVS.

We always refer to previous studies for adverse effects of therapeutic agents used. When a group is given a treatment for the first time, we initiate the study with a small number of animals (n=2-4) which is closely monitored before extending to a larger number. Where possible we use the least invasive method of drug administration.

We will receive training from experts within our institution to apply endoscope guided methods for the injection of cancer cells. This represents a new technique with reduced adverse effects when compared to laparoscopic approaches.

Welfare handling methods will be applied throughout the project. Researchers are trained for the handling of each animal model and signed off only when proficient in the clinical signs of the models.

Animals are housed in a dedicated facility proactive with environmental enrichment and receive anaesthesia and analgesia as appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

AWERBs and NC3Rs

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with Home Office and NC3R guidance through the information published within their website but also by attending meetings and staff forums. We continually review our processes and take advice from the Named Veterinary Surgeons, Named Training and Competency Officer and Home Office Inspectors.

136. Studying cognitive function in animal models of brain disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

Memory, Animal behaviour, Brain disorders, Schizophrenia, Negative symptoms

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify and assess the effects of drugs and other treatment interventions (e.g. exercise and environmental enrichment) in preventing or rescuing memory and other behavioural deficits in validated rat models for symptoms of brain disorders.

To determine how pharmacological agents and non-pharmacological treatments work. To develop new tests and new models for mimicking the symptoms of brain 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?

It is important to undertake this work to address the unmet clinical need of memory and behavioural dysfunction in a number of brain disorders. Currently there is no licensed medication for the treatment of memory problems and negative symptoms in schizophrenia. Also the medication available to treat the symptoms in other brain disorders do not work very well.

What outputs do you think you will see at the end of this project?

Scientific publications - developing new information around the causes of memory impairment and new targets for people working to develop better tests and treatments.

Testing new treatment plans in this project could lead to new medicines for clinical trials in people with brain disorders.

New tests and models for mimicking the symptoms of brain disorders.

Who or what will benefit from these outputs, and how?

The scientific community and general public, through furthering knowledge, and improving understanding. The pharmaceutical industry, and drug discovery groups, as we will assist in providing new knowledge around new treatment targets. We have close links with the pharmaceutical industry and will inform their drug discovery plans through these links and via publication of our findings.
Patients, carers and the NHS as ultimately this work will lead to new improved medicines for patients.

How will you look to maximise the outputs of this work?

We are committed to the publication of negative and positive results. We regularly present our findings at national and international conferences and publish in high ranking scientific journals. Further to this we have a strong background in public engagement, regularly presenting at SciBar events and taking part in events for the public. Some of the work carried out is in collaboration with the pharmaceutical industry and experts from academia. We routinely use tissue for other complementary studies (e.g. electrophysiological studies that utilise techniques to look at how brain regions talk to each other) and post-mortem analysis (looking at relevant brain markers) ensuring we are always developing our knowledge of the model and its relationship with human findings.

Species and numbers of animals expected to be used

- Rats: A maximum total of 9500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
Much is already known about brain anatomy and chemistry, memory and how the brain works to control complex behaviours in adult rats. We have many years of experience in studying complex behaviour in rats, all our current tests are validated for rats and our

tissue analysis techniques and methods are validated using rats. Many of our tests e.g. for complex memory and mood are fully validated for adult rats and not for other rodents.

Typically, what will be done to an animal used in your project?

Rats may be given a drug treatment by injection, or an environmental treatment such as voluntary exercise in running wheels. This may be carried out before or after the administration of compounds that induce memory changes and changes to brain circuits, e.g., phencyclidine (PCP), a psychotomimetic drug that mimics some of the symptoms of schizophrenia in both rats and man, such as hyperactivity, issues with memory, decreased motivation and also not wanting to interact with others.

A small blood sample may be taken from the tail vein at specified times to measure drug levels and/or biomarkers.

Some rats may be mildly food deprived and trained in food motivated tasks, the effect of treatments as described above will be measured to determine their effects on cognition and other behaviours such as activity.

Alternatively, some groups of untreated rats will be assessed in tests of memory and behaviour using long delays between trials to measure forgetting, or task manipulation to make the task more difficult, resulting in an increased demand on the brain systems.

What are the expected impacts and/or adverse effects for the animals during your project?

The administration of phencyclidine (PCP) and other drugs may produce mild short-term pain from the injection site and also cause mild short-lived changes in behaviour such as an increase/decrease in activity.

Behavioural techniques are generally not stressful and can, in certain cases, be considered as enrichment.

Some animals will be placed on mild food restriction during testing and lose up to 10% of their free feeding bodyweight.

Some rats will have blood samples taken which may cause mild short-term stress from restraint and pain from the needle insertion.

At the end of the study, or as part of the experimental procedure rats will be killed humanely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected maximum severity for the three protocols in this license is moderate. Approximately 80% of the rats used will reach moderate severity and 20% will reach mild severity.

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to measure the control of the brain on behaviour in cell systems. Much is already known about memory and how the brain controls complex behaviours in rats, so studies using live animals are essential to obtain a greater understanding of normal and abnormal states and to test the effectiveness of new treatments. Currently, this work must involve the use of whole animals as studying the effects on behaviour is a central feature of the project and as to date, there is no suitable alternative to using rodents for these types of studies.

Which non-animal alternatives did you consider for use in this project?

Where possible we perform studies using in-vitro cell models to aid our understanding or how cells in the brain work.

We also undertake a number of studies using human post-mortem brain tissue to help work out similarities and differences with our animal studies.

However, studying behaviour and how memory, mood and social impairment is related to brain disorders is extremely complex and cannot be fully studied using in-vitro preparations or computational approaches.

This work therefore must require the use of animals as behaviour and testing new treatments is the most important part of the project.

We only test new compounds that have been carefully selected using in-vitro screens to ensure only the most promising drugs go into our animals.

Furthermore, we first conduct pilot studies with each new compound, starting at the lowest dose to check for any potential side effects prior to larger studies.

Why were they not suitable?

Currently there is no suitable alternative to the use of animals for behavioural research.

It is necessary to use animals to model the complex memory and social deficits seen in brain disorders such as schizophrenia.

In-vitro preparations or computational tests cannot be used as they lack the required complexity due to insufficient biological data.

We will carry out in-depth analysis on tissue samples from animals as we are searching for biological and behavioural changes induced by our model and 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?

We have 20 years' experience in developing and refining this model and behavioural tests so that the fewest numbers of animals are used. Estimations are based on the numbers of rats used in studies in previous years. In 2020 we increase the estimated number of rats used as study numbers had risen due to demand from pharmaceutical company clients.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken advice from an external, approved statistical expert, who has assessed our power analysis calculations to ensure that we have minimum number of animals for the maximum statistical power.

We have lots of experience of analysis of these datasets from our previous work and in consultation with other external statistical experts and colleagues within the Pharmaceutical Industry and at this University. We have a member of the team who works with the NC3Rs Experimental Design Assistant to help us to design robust animal experiments.

Where appropriate we minimise the overall numbers of animals used by re-using the same animal in different behavioural tests, and where appropriate, testing more than one drug in the same animal.
Numbers will be further reduced by using repeated measures designs where possible. Re-using the same animals is only carried out following veterinary examinations "fit to remain" immediately following a treatment/procedure and a second veterinary examination prior to re-use "fit to be re-used".

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use the literature and pilot studies in small numbers of animals to guide us when setting up new behavioural tests, ensure animal stress is kept to a minimum, ensure animals are used to the testing arenas before studies begin and we also provide our researchers with excellent training.

We will monitor the reliability of our studies closely and upon generation of new data we can alter group sizes as appropriate and in consultation with statistical experts.

We are working closely with colleagues to develop behavioural tests that improve data yield to reduce animal numbers further by minimising the potential negative effect of animal handling in our studies.

We have a number of behavioural tests that require minimum training so where appropriate we minimise the overall numbers of animals used by re-using the same animal in different behavioural tests, and where appropriate testing more than one drug in the same animals.

We routinely collect blood and brain post-mortem tissue and share tissue from our animals with other research groups, which optimises the numbers of animals used and will maximise data output from every 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.

Animal Models.

We use rats as we have detailed knowledge of their behaviour and brain organisation, which shows remarkable similarity to humans. Our rat models of human diseases show symptoms such as memory loss and subtle brain changes that are very similar to human patients. All our current tests are fully validated in rats and our tissue analysis systems are validated using rat brain.

We will use a sub-chronic (7 day) dosing schedule of psychotomimetics (e.g. phencyclidine) to produce long-lasting behavioural deficits in rats, which can only be seen when their memory and social behaviour is challenged, the effects are very specific to memory deficits with low impact to the welfare of the animals. We also use longer time-delays between experimental trials in some of our tests which allows the animals to naturally forget what they had previously learnt.

Methods and monitoring.

Welfare is very important for successful experiments and we continually aim to refine our techniques and practices. Our behavioural tests, which we often use to longitudinally assess our rats in a number of behavioural tasks, are not stressful to the animals, and could be considered as enrichment. We aim where possible to refine animal welfare for our laboratory rats.

In our recent experiments we started to investigate the use of playpens for cages of animals as a method of improving welfare in animals used in long term behavioural studies. These studies are ongoing and in collaboration with colleagues in academia. Making sure the playpen system works effectively could lead to a method that can be incorporated in to other animal facilities housing rodents and provide additional environmental and social enrichment.

We use a validated monitoring system to assess animals for adverse effects for any new treatment, which greatly reduces the number of animals that could experience a potential adverse effect. For animals that do receive a new treatment in this way, careful continual monitoring ensures termination is rapid if an endpoint is reached. This procedure ensures rapid decision making and actions that minimise any potential suffering to that animal. Before we test a new drug for a pharmaceutical company in one of our behavioural tasks, information is shared to prove that the compound has previously been checked for safety and the dose ranges we aim to use do not produce adverse side effects, thus ensuring that adverse effects in our studies are further minimised.

None of the models that we will use are expected to experience severe side effects; however, if seen in any animal it will be humanely killed.

Why can't you use animals that are less sentient?

An adult rat is superior for our studies compared to a less sentient species or a more immature life stage because the physiological systems involved in learning and memory have been so extensively studied and widely published in this adult animal.

We are unable to measure complex behaviour patterns in immature life stages, less sentient species or in a terminally anaesthetised animal. We have selected adult rats as their social structure enables detailed analysis of social behaviour. Furthermore, this work must entail the use of the whole animal as behaviour is a central feature of the project. If any relevant, non-animal alternatives become available during the course of this project, we will aim to implement them in our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have started to study the effects of time spent in playpens on rat behaviour with the aim of possibly introducing this to our experimental animals and highlighting the effects of this enrichment to other researchers and colleagues. We have also conducted a pilot study to assess the effects of playful handling of rats, which shares some similarities to the rat tickling technique, on behavioural outputs.
Our ongoing studies are comparing the positive effects of exercise on memory in the form of voluntary wheel running in rats.

Refinements include habituating rats to new behavioural test arenas in home cage groups to reduce any potential stress when habituated singly.

Animals undergoing dosing procedures are removed from the main holding room into a separate room to prevent the induction of stress to stock animals and other experimental animals due to vocalisations.

In our longer-term studies such as reversal learning, rats are placed onto soft paper bedding instead of standard wood-based bedding to prevent the risk of developing hock sores.

Further refinements include reducing the maximum total number of injections of pharmacological compounds from 28 to 21 days and also reduced the frequency of injections over the 21 day period to once per day instead of twice per day (after day 7). Recently we employed an expert in rodent handling and procedures to provide a training/refresher course in animal restraint and oral gavage dosing for our research

technicians. This expert training ensures improved handling, reduced restraint and improved dosing techniques resulting in reduced stress to the animals and improved animal welfare.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We consult with other leaders in the field of behavioural research to implement any improvements in animal welfare and care. We have access to the extensive library of NC3Rs resources which includes guidelines, practical information, links to themed hubs and publications, other online resources, and video training materials. We routinely attend appropriate events organised by the NC3Rs and our local animal facility. We follow the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) for guidance on how to plan and report animal experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We attend, and encourage other members of the team to register for the regular 3Rs symposiums and webinars organised by the NC3Rs. We have taken advice from our local NC3Rs manager, on implementing environmental enrichment using play pens and we are currently collaborating with researchers at others Universities, with the aim to apply for an NC3Rs project grant to study the effects of play pen enrichment on rat welfare and behaviour.

137. The physiological roles of phosphatidylinositol 3-kinases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cell Signalling Pathways, Genetically Engineered Mice, Translational, Disease, Therapeutics

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to investigate the roles of phosphoinositide 3-kinases (PI3Ks), a family of intracellular signalling molecules that transmit information from the outside of the cell to within the cell. This work has led to approved drugs and continues to inform ongoing drug development.

Our research focuses on diseases such as cancer and immune and metabolic dysregulation (where PI3Ks are not functioning normally) with the aim to develop therapeutics to correct them. We are also using approaches to activate PI3K for therapeutic benefit, such as in cardioprotection or wound 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?

The experiments performed under this licence will not only contribute to the understanding of how signalling pathways operate in cells, and when disrupted, cause disease, but also allows us to develop therapeutics to alleviate these diseases. So far, we have found that disrupting the pathways controlled by the PI3K gene family (which act by transmitting signals from the outside to the inside of the cell) may result in, or protect against, diseases of metabolism, immunology and cancer. By carefully studying these disorders we can develop or fine-tune the design of therapeutics to mimic the genetic inactivation or genetic activation, whichever is best, to correct these disorders.

As a result of our work and those of collaborators, drugs have been developed that have now been approved or in clinical trials and are helping to treat people with cancer and immune dysfunction. We hope to develop more therapeutics through continuation of this work under this licence.

Other than treating cancer when it has developed, it would also be desirable to prevent cancer from arising in the first place. Given that medicines that block PI3K have now become available, we are now also using these drugs to test if they could be used to prevent cancer from developing. We hereby use a mouse model that faithfully mimics a human cancer predisposition syndrome driven by a PI3K- activating mutation in their genes. We postulate that the PI3K inhibitors will counterbalance the PI3K- activating mutation, and thereby prevent cancer from developing.

What outputs do you think you will see at the end of this project?

We aim to produce the following outputs whilst working under this project: -

Increased understanding of PI3K pathways in disease, including disorders of cancer, immunology, metabolism and development.

Translate this understanding into novel therapeutics to treat or prevent these diseases.

Publish our findings in peer reviewed journals and contribute to the body of scientific insight into the basic science of PI3K and its translational potential.

Use the knowledge gained to train a new generation of scientists.

Who or what will benefit from these outputs, and how?

The impact of the outputs in the short term will be the increased understanding of the role of PI3K molecules in cellular signalling pathways, in disease, and its translation into novel therapeutics to treat these disorders. This will be disseminated to the scientific community through collaborations, peer- reviewed publications and talks at conferences. The long-term benefit would be clinical trials of the novel therapeutics in humans and ultimately alleviation of the disorders mentioned.

How will you look to maximise the outputs of this work?
The studies undertaken under this project licence are often collaborations with other research groups, across the UK, and Worldwide. We have constructed over a dozen novel genetically engineered mice over the last 20 years that have been shipped to many Worldwide Academic and Pharmaceutical Institutions and have now all been deposited in

publicly accessible repositories. The project licence holder and his fellows continue strong ties with many of these collaborators, some of which were previous postdocs in the licence-holder's laboratory, continuing the work that they started during their training as independent scientists and passing on the new knowledge gained to a new generation of scientists. As the field of collaborations is large, approaches to the work can be quickly and easily refined, negating previous unsuccessful approaches, so that work can be channelled down more fruitful paths.

Once we are sure that the work has scientific validity, the data will be peer-reviewed by established scientists in the field, then disseminated through publication in scientific journals, talks at conferences, through general communication with collaborators and ultimately through the press to the public at large. In the case of important findings, we effectively engage with relevant Press Offices who provide effective communication of our findings to the wider world in print and on-line.

Species and numbers of animals expected to be used

- Mice: 24,000
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are used in this study because they resemble humans in many ways, anatomically, physiologically and genetically. Unlike lower genetic model organisms such as flies and worms, mice share all eight PI3K genes with humans which when mutated can cause or protect against disorders such as cancer or inflammation. The administration of therapeutics to mice can be done in nearly identical ways that would be done in humans, therefore providing good insight and the groundwork for clinical trials. We are increasingly resorting to administration of drugs in the food, effectively mimicking the administration of an oral pill, with drug exposure over at least 12hrs (given that the mice do not eat when the lights are on).

Except for the creation and breeding of genetically altered mice, adult mice will be used for the majority of protocols where ageing, metabolism, cancer and the immune system are being investigated. This follows from the fact that these diseases usually present themselves in humans in adulthood. The exceptions being (1) overgrowth syndrome, where clinical signs are present from conception and therefore all life stages will be investigated; (2) mice in which genetic alteration leads to embryonic lethality. Investigating the biology of these embryos has been very powerful to uncover new biological functions of PI3Ks.

Typically, what will be done to an animal used in your project?

Within this project licence, the following procedures will be used in order to achieve our aims: -

In the case of the creation, breeding and maintenance of genetically altered mice, mice will either be sought from commercial suppliers or created ourselves using standard well-established procedures. Female mice will be superovulated by intraperitoneal injection of hormones such that they generate many hundreds of embryos that can be removed from the uterus by a Schedule 1 cull. These embryos will then be manipulated in vitro and genetically altered. A recipient female mouse, in parallel will be made pseudo-pregnant by mating with a sterile male mouse that has been made infertile by surgical means. The altered embryos can then be engrafted surgically into this female, where the embryos can develop to full-term, be born, weaned and maintained for further breeding or used for experiments described below. These embryos can also be archived by cryopreservation such that, if a time comes when the strain is not required, live mice will no longer be needed for breeding and maintenance.

Mice that have inducible genes that can be switched on or off, will be given inducers such as tamoxifen, either by intraperitoneal injection, or administration by gavage that enables gene expression control. Typically, this will be done once, up to several times, over consecutive days, before mice are engaged in the relevant experiment.

Novel genetically altered mice will be subjected to broad phenotypic characterisation by a dedicated mouse pathologist in the fields of immunology, cancer, ageing, metabolism and development to determine whether gene inactivation of the PI3K pathway can cause or alleviate disease and if therapeutics, based on genetic alteration, can correct any problems.

Therapeutic substances will typically be administered in the food or water for the entire life of the mice but may also be administered daily for shorter periods, for up to a few months, through routes such as oral gavage (forced feeding by a tube inserted into the stomach, or injection).

During ageing studies, mice, including those that develop cancer, will be kept for their entire lifespan; that is, up until the humane endpoint - for example, maximum tumour size or the limit of acceptable deterioration of health through observable body scoring. Therapeutics may be given, mixed in with their usual daily food and at specific time points mice will be culled by schedule 1 or non- recovery anaesthesia so that blood and tissue samples can be collected for examination. These can then be processed and analysed to determine whether genetic alteration or administered therapeutics have had any effect on the progression of the disease. Blood samples may also be taken periodically from the tail vein of these mice for pharmacokinetic studies, and also following insulin or glucose intraperitoneal injections to evaluate if these strains develop any metabolic defects. Measurements of blood parameters may be done up to 4 times during the lifespan of a mouse.

One novel therapeutic strategy that we are developing is obtaining bone marrow cells from normal mice and injecting them into irradiated mice that are genetically predisposed to develop cancer to see if this helps adjust the immune system to fight cancer progression. After engraftment of normal bone marrow cells, mice will be kept for their entire lifespan and investigated as above in ageing studies.

Cancer predisposition: we are using a mouse model that is predisposed to cancer, as the result of partial inactivation of a gene that downregulates PI3K activity. This mouse model faithfully mimics a human cancer predisposition syndrome called PHTS (PTEN Hamartoma Tumour Syndrome). Given that PI3K inhibitors have now become available, we are using these drugs to explore if they can prevent cancer in this mouse model.

Immune-deficient mice may also be injected with cancer cells, on one or two occasions, for example subcutaneously (under the skin), or by intraperitoneal injection (into the abdominal cavity) and again treated with therapeutics, usually in the food. These studies generally last 2 to 3 months and allow us to study the treatment and progression of human cancers and the role of the immune system in cancer. There now exists a fair number of immune-deficient mice where various compartments of the immune system have been removed. By comparing the growth of tumours in these different mice and the added effect of therapeutics in the food we can evaluate the effect of each immune compartment in isolation and the effect of therapeutics on these immune cells. That is, can we manipulate and enhance certain aspects of the immune system to better control or prevent tumour growth.

Experiments with subcutaneous tumours may also consist of dual therapeutic regimes, such as the use of radiation and therapeutic substances administered through the food or by daily gavage for several weeks. Such therapeutic regimes may not only be additive but may have a synergistic and therefore beneficial effect on tumour progression.

Cell signalling pathways are known to be involved in the way cells migrate and divide, a hallmark that is abnormal in cancer cells that may help them metastasise (migrate) to other organs of the body. It is known that some cancer cells may operate in a similar fashion to cells involved in wound healing, helping them not only to propagate through the primary tissue sites, but to move in and out of blood vessels such that they can spread to other organs. One method that we can use to investigate this is to cause up to two small skin wounds in a mouse and see how the gene inactivation or therapeutics alters the progression of the healing process over several days to a few weeks. Another method that can be utilised is ischemia reperfusion (tissue damage caused when blood supply returns to tissue after a period of absence or lack of oxygen), where the femoral artery, the main blood vessel in the leg is ligated, causing injury to the surrounding tissue by lack of nutrient and oxygen delivery. Again, mice will be observed for several days to a few weeks to assess how the injury resolves back to normal tissue.

This method also allows an insight into the roles of oxygen deprivation and blood vessels in the progression of wound healing and cancer metastasis. In the case of metastatic mouse cancer models, such as those of the breast, subcutaneous tumours may be surgically removed and mice kept for several months to assess the effects of gene inactivation or therapeutics on the degree of metastasis. This allows us to develop therapeutics for relapses in breast cancer, where even though primary breast tumours may have been successfully treated or removed, the patient later goes on to develop lung metastasis several months later.

As we know that genetic or therapeutic alteration of the PI3K pathway can enhance the immune system to treat the progression of cancer, we are interested in exploring if this immune manipulation could also be beneficial in treating infections or allergies where the immune response is required or behaving abnormally. During these studies, for example, mice will be injected with live or attenuated pathogens and their immune response measured by taking blood samples and tissues at specific time points. Therapeutics may also be administered typically in food or water, but may also be administered by gavage, or intraperitoneal injection. Such experiments would not usually last more than a week or two.

Colitis, a disease of the intestines, caused by abnormal immune function leading to chronic inflammation of the colon can in some instances lead to colon cancer. However, in

some strains of PI3K mutant mice where colitis is observed, progression to cancer has not been observed. It would be interesting to investigate why this is so by 'pushing the system' such that colon cancer does occur. To do this, mice will be administered with a carcinogen by intraperitoneal injection and colitis induced with a substance in the drinking water. Mice will be kept for several weeks, and blood and tissues analysed after culling by non-recovery anaesthesia or a Schedule 1 method.

PI3K mice do not develop diabetes, even though abnormal PI3K signalling alters the way glucose is metabolised. In order to investigate this further we will use a model of diabetes that is induced using a chemical called streptozotocin that is added to the drinking water, food, or administered by intraperitoneal injection. Mice will be kept for several weeks to further assess if the PI3K pathway resists the progression to diabetes.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts and/or adverse effects of these studies on animals would include: -

Weight loss - gradual over several weeks or more quickly over a few days. Not more than 15% deviation from the start of experiment.

Deterioration in general health caused by progression of disease such as inflammation or cancer as assessed by body scoring. For example, staring coat, laboured breathing and hunching that would last for no longer than 24hrs.

Tumours, inflammation or immunological agents may cause long lasting mild pain over several days, or moderate pain up to 24hrs.

Mice may be predisposed to, or subjected to developmental, metabolic, immunological and disorders that lead to cancer. These would develop over the course of the lifetime of the animals, most remaining subclinical such that the animal experiences no pain. However, some genetic models may develop mild distress slowly over a few weeks and others moderate distress up to 24hrs.

Expected severity categories and the 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 creation, breeding, and maintenance of genetically engineered PI3K mice, should generally cause no more than sub-threshold severity in the case of mice that produce phenotypes (the observable physical properties of an organism) that have no clinical manifestation. In the case where phenotypes show clinical signs, they are generally mild and in the case of harmful mutants where tumours arise, mice will be culled before the onset of clinical signs such that breeding mice will not experience severity greater than mild or in a rare case, moderate.

In the cases where surgery is required in the creation of genetically engineered strains, or more the implantation or removal of tumours, mice will experience moderate signs of distress.

In the case where mice are injected with inflammatory substances, injected with cancer cells or develop spontaneous phenotypes with clinical manifestation, then mice will suffer no more than mild signs of distress if the scientific endpoint is reached before the humane endpoint. We should be able to generate and collect must of our data under a mild severity. However, if further data is required, then mice will be allowed to reach a humane endpoint where they will experience moderate signs of distress for no more than a day or two. This will also be the case for metabolic and developmental studies.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The reasons why we require live animals for our studies are discussed below.

Mammals such as mice and humans have 8 distinct PI3K genes, which determine the functioning of the PI3K signalling molecules, belonging to 3 separate families. Other widely used model organisms such as Drosophila and C.elegans only have a small subset of these genes (for example flies have 3 PI3K genes) and are therefore not suitable as model systems to study human biology. The complex disease processes that we investigate also cannot be modelled appropriately in these lower organisms.

Genes that regulate cancer, the immune system and metabolism are generally pleiotropic (have more than one function), often interact with many other genes and therefore may be relevant for many organs and biological functions. Therefore, in order to study a disease caused by a genetic alteration, the whole complexity of an organism is required. In addition, the systems that we would like to study such as immunology and development require thousands of genes.

In order to study tumour formation and especially metastasis, the whole animal is required. Specific cancers affect specific organs and often metastasize to other organs in unpredictable ways. Therefore, animals are required in order to dissect the roles of PI3K isoforms in specific cancers.

Therapy using inhibitors often give promising results in cell-based assays, only to fail in vivo. This is because the pharmacokinetics (the time course of drug absorption, distribution, metabolism, and excretion that determines its potency) is often dependent on route of administration, metabolism and many other factors that do not exist in cell culture. Therefore, live animals are required to make an accurate assessment of pharmacokinetics of the combined inhibitors, effect on tumours, or their immunosuppressive effects.

During the growth of tumours, a process called angiogenesis allows the formation of blood vessels from pre-existing vessels. This, for example can occur during tumour formation or during development. Both systems require the whole animal.

Rescuing mutant phenotypes and whole animal gene expression, such that the animal reverts back to its normal state, also requires the whole animal; there are no in vitro substitutes available.

When it comes to the study of metabolic regulation, many organs, such as fat, muscle, brain, pancreas often act together in relation to environmental factors. These factors also affect lifespan. Therefore, live animals are required in order to study metabolism and lifespan.

Which non-animal alternatives did you consider for use in this project?

In vitro studies using cell-based assays will be used initially to determine whether PI3K signalling pathways are operating in the organs or cells under study and whether therapeutics or modulators affect these pathways. If we have reasonable evidence to suggest this is the case, then in vivo models will then be carried out.

We are in the process of developing organoids, a mass of tissues and cells that resemble an organ - a miniature organ that you can propagate in vitro. These may negate some of the arguments that a whole mouse is required for some studies. For example, we are developing a system to evaluate cell signalling in small and large intestinal organoids, which will also involve inducing inflammation and testing of inhibitors.

Why were they not suitable?

Cell-based assays often give a good indication of how a specific cell responds to a specific inhibitor. However, inhibitors often perturb other systems or organs other than its target, and in order to evaluate these effects the whole animal is required. Also, the whole animal is required to assess how inhibitors affect for example cancer metastasis. Immunology, cancer, metabolism and related diseases are difficult to replicate in laboratory-based models such as tissue culture and organoids. This is because these diseases are the result of complex interactions between different 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 numbers of mice we would like to use for the experiments would primarily have been based on previous experiments or those performed in the literature or by collaborators. Numbers for experiments have been advised by a statistician who we communicate with regarding experimental design of these studies. In this way we are confident to produce meaningful scientific data with the least number of mice possible.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A specific mutant mouse will only be created if no other such mouse line exists. This will prevent unnecessary duplication of work and mice.

Mice will be bred on a need-for-experiments only. In other cases, the minimum number of mice will be kept in order to maintain the colony. In some cases, where a particular strain of mouse is not needed for long periods of time, mouse embryos will be kept as frozen stocks thus keeping mouse numbers to a minimum. These frozen stocks are also used for shipments to collaborators.

To prevent variation in data, technicians skilled in the techniques required will be used, along with high quality reagents and cells. For experiments, age and sex-matched animals will be used to discount bias from aging and gender. Inbred strains will also be used to discount the effect of genetic variation as would be the case in outbred strains. To further reduce variability and unwanted bias occurring in the data obtained from the experiment, mice from each cage will be randomised such that they have an equal chance of receiving a given treatment; each treatment being administered blindly. Data will be collected from all mice in an identical way.

Expression of all genes and therapeutics will first be tested in vitro to assess their suitability for in vivo work.

The NC3R's ARRIVE guidelines and EDA (Experimental Design Assistant) will be consulted before any experiment is designed or carried out.

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 extensive databases of our mouse lines and record every breeder set up, and all pups born. We also have a good idea of the average number of pups born per strain and genotype ratios. This prevents overbreeding when maintaining lines and helps us to breed the correct number of mice for experiments from parents with the correct genotypes.

After the creation of a new mutant strain of mouse, mice are analysed by histology of most tissues by an expert mouse histopathologist, blood analysis and biochemical parameters, as a way to find phenotypes in mutant mice. It allows us to gauge the degree of disease in a particular mutant mouse and allows us to plan what experimental protocols, if any, are required to evaluate the mechanism of disease. It gives us an idea what group sizes are required for live experiments; for example, if the type of disease is highly variable or not fully penetrant in all mutants, then large group sizes will be required and vice versa.

One example of an experimental approach would be to observe tumour growth in mutant PI3K mice and their wild-type littermate controls. We can then determine, by comparing to wild-type controls whether PI3K isoforms ameliorate or exacerbate tumour formation or metastasis. Initially, pilot studies containing small numbers of mice, (not more than 5 per group) will be carried out to assess the aggressiveness of each tumour type with each isoform and the time taken to reach the humane endpoint of the study and whether sufficient scientific data has been collected within this time frame. This will be done in accordance with previous work and data that is accessible in the scientific literature or from collaborators. From the data produced in the pilot studies we can then design larger scale experiments if necessary and estimate group sizes based on statistical tests. If any PI3K isoform ameliorates tumour formation or prevents metastasis then we can then precede to therapeutic studies. When it comes to dosing animals with therapeutic compounds, we often use factorial designs; that is, often, more than one drug is tested, using one control group for all therapies, and often one therapeutic being a positive control.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be performing experiments that model animal diseases such as cancer, those that affect metabolism, or the immune system. These diseases are often interlinked and may also affect how development may occur.

The models we chose will use only minor technique's that will cause brief transient distress such as injections or oral gavaging. In the case of administering therapeutic agents such as PI3K inhibitors or activators, the drug will generally be given as part of their diet, thus avoiding the need for injections or gavaging.

Tumour models will generally be restricted to non-metastatic models where the tumour remains localised in one place, is unlikely to invade and harm another organ and will not be allowed to grow beyond 1.4cm^2 in diameter and therefore unlikely to affect the animal's ability to eat or drink. Spontaneous models will often be used, so avoiding techniques such as injecting cells to induce tumours.

For metabolic and ageing studies, mice will be restricted to the number of procedures that any mouse can receive in a lifetime such that a moderate degree of severity is not breached.

We will be performing experiments that model animal diseases such as cancer, those that affect metabolism, or the immune system. These organ systems are interlinked and together affect the development of disease or therapy response.

The models we chose will use only minor techniques that will cause brief transient distress such as injections or oral gavaging. In the case of administering therapeutic agents such as PI3K inhibitors or activators, the drug will generally be given as part of their diet, thus avoiding the need for injections or gavaging.

For immunological studies that require the administration of pathogenic agents, this will generally be restricted to using only attenuated forms of pathogenic agents or their analogs such that much milder infections are induced that are unlikely to be severe or fatal. In addition, these studies will generally be completed within several days.

Why can’t you use animals that are less sentient?

Mammals such as mice and humans have 8 distinct PI3K genes, belonging to 3 separate families. Other widely used model organisms such as Drosophila and C.elegans only have a small subset of these genes (for example flies have 3 PI3K genes) and are therefore not

suitable to use as model systems to study human biology. The complex disease processes that we investigate can also not be modelled appropriately in these lower organisms.

In order to study cancer, metabolism and immunology we require to model diseases in an organism that is very similar to man such as mice which also have the lowest neurophysiological sensitivity of vertebrates.

The mouse allows processes such as metastasis, the connection between the immune system, metabolism and cancer to be studied more accurately which wouldn't be achievable in less sentient organisms. Adult mice will generally be required due to the timescale of progression of diseases such as cancer and the maturation of the immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinements that are included for the protocols in this project licence are described in the following paragraphs.

Novel mouse strains will be observed daily to monitor the onset of any phenotype; weight, piloerection, abnormal breathing rate, skin turgor, behaviour, eye discharge and mobility will be checked as indicators of suffering. Any deviations from the norm, including the timing of the onset of suffering, will be monitored and used to predict future occurrences. If sufficient scientific data is obtained before the humane end point of the experiment is reached, mice will be culled to prevent further suffering.

In addition, blood and tissue will be collected from culled mice and analysed to determine phenotypes. Additional monitoring can then be implemented as better understanding of the phenotype and the timing of the humane endpoint is reached. Mice will be observed, and a body score taken in accordance with Appendix 5 or 6, at least twice weekly, at around 3 days apart, or with greater frequency as the humane endpoints approach. This will enable the experimenter to assess the level of pain and distress that the animal is experiencing in addition to other welfare indicators specific to each protocol and to refine these if necessary.

During cancer studies, animals will be observed for signs of tumour growth for their entire lifespan by blood sampling and calliper measurements of external tumours. For animals with internal tumours, pilot experiments will be carried out with routine necropsies taken to determine the growth rate of the tumours and its effect on the welfare of the animals. This will enable the timing of scientific and humane endpoints to be determined.

“Guidelines for the Welfare and Use of Animals in Cancer Research,” by Professor Paul Workman et al, under the auspices of the UK's National Cancer Research Institute (NCRI) will be followed.

Therapeutics will usually be given to mice in food or water thus negating the need for handling, repeated injections or gavaging and undue stress to the animals resulting from these invasive procedures.

When mice are given therapeutic substances, such as PI3K inhibitors, that have been added to food or water, food or water intake will be monitored, and mouse weights recorded daily to ensure that mice are drinking and eating normally. If mice lose up to 15% bodyweight or show signs of dehydration as described above mice will be culled.

Compounds given to drinking water may be sweetened with 1% sucrose to improve palatability.

Initially, any topical therapeutic compound will be scrutinised for its suitability to be skin permeable or dissolved in an ointment that would facilitate its absorption into the skin. To minimise this risk, the dose of therapeutics used will be based on what has been effective by other routes of administration, from our own data or those of collaborators without causing complications. The ointment used containing the therapeutic will be based on those known not to cause any adverse effects in humans and animals. If any soreness is noted, for example flaky skin or redness, or if there are any other adverse effects, the application will be discontinued and advice from the NVS sought.

Suitable administered volumes and doses of therapeutics, immunogenic substances or cells will be determined by referring to our own, collaborators and published data. If suitable doses and volumes are not known, then pilot studies will be initially carried out. Pilot studies will consist of injecting one animal with a low dose and increasing the dose stepwise in other animals, until the desired scientific endpoint is reached, or before a moderate degree of suffering is reached, whichever is lowest – this will dictate the maximum dose for the experiment. The effects of each dose will be evaluated before adopting a stepwise approach to higher doses, such that the humane endpoints are not breached.

In all protocols, animals will be weighed and body-scored at least twice weekly, at around 3 days apart, and with increased frequency as the endpoints approach.

Other than in terminally anaesthetised animals, dosing and sampling procedures will be undertaken by a person competent in the relevant technique, in accordance with good practice using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm and are the minimum compatible with the scientific objectives.
The latest procedures that cause the least suffering to mice will be used to replace older, outdated methods. Unless otherwise stated in the protocol, all substances will be administered in accordance with “Refining procedures for the administration of substances by Morton et al Laboratory Animals 2001 35 1-41,” in accordance with Appendix 4.

For protocols that require surgery, procedures will be carried out aseptically and post-operative analgesia will be given after surgical procedures to manage anypain.

If protocols require anaesthesia, this will involve agents and methods suitable for the species as advised by the NVS. Depending on the anaesthetic regime used, animals will be ambulatory within a few minutes to 1hr of the end of anaesthesia. Supplemental heat and analgesia will be provided as advised by the NVS during surgery and recovery. Any animal which has not resumed normal mobility, eating and drinking by the end of the working day of surgery will be killed.

Where the immune status of the animals or a procedure such as irradiation might compromise health, they will be held in a barrier environment and/or given antibiotics to minimise the risk of illness due to microorganisms present naturally in the environment.

If a procedure, such as those that induce diabetes, is likely to cause dehydration over a short period of time, gel formulations, such as hydrogel, may be given to mice to help keep them fully hydrated. These mice will be given wet food or food gels.

For diabetic mice, due to the possibility of increased drinking and urination, cages or bedding will be changed twice weekly, at around 3 days apart, or when excessive soiling has occurred. Mice may also be supplied with 10% sucrose in the drinking water overnight in case hypoglycaemia occurs.

Several breeding and experimental scenarios may arise where mice find themselves individually housed. For example, male mice born without brothers in a litter that are required for breeding or experiments at a later date. Also, during experiments mice are often culled at different times, depending on when the scientific or humane endpoints are met for each mouse, often leaving mice individually housed.

In order to minimise this from occurring, weaning will be managed to avoid singly housed animals wherever possible and mice that are of no scientific interest or are not required for breeding will be culled. In cases where singly housed males are to be kept for experimental or breeding purposes, post- reproductive females will be sought as cage companions.

Where the immune status of the animals might compromise health, they will be held in a barrier environment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best guidance practice published on websites such as NC3Rs and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?
We will ensure to use the most refined methodology by: -

Literature searches.

Checking websites such as NC3Rs and ARRIVE guidelines.

Frequent communication with collaborators in Industry and Academia who have carried out similar work.

Checking with the NVS, NACWO and BSU staff before any procedure is carried so that we can implement these techniques effectively.

138. The role of redox signalling in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Protein tyrosine phosphatase Signalling, Cancer, Redox biology, Biosensor, Immunology

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to increase our understanding of the molecular signals that allow the cells in our bodies to talk to each other and work together, that when deregulated can cause or worsen diseases such as cancer, or promote damage associated with limiting healthspan.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 better understanding the biological mechanisms underpinning human health and the causes of disease will allow us to improve the way we treat disease in the future. It will reveal new strategies or therapies with as few undesirable side-effects as possible.

What outputs do you think you will see at the end of this project?

The expected benefit from this project is that we will gain a better understanding of the complex molecular and cellular mechanisms that underpin a healthy lifespan that - when deregulated - can cause or exacerbate complex diseases such as immuno-deficiencies and cancer, as well as age- related decline. Our findings will be published in open-access

journals. In the long term, the knowledge generated from this project might contribute to the development of more effective drugs.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries are the scientific community and the interested general public who will gain new understanding from our open access publications, presentations and conferences and public engagement activities. In the longer term, the pharmaceutical industry may benefit by exploiting the knowledge generated to develop new therapies. Eventually, society as a whole may benefit from the improved health brought by such interventions.

How will you look to maximise the outputs of this work?

We will publish our findings in open-access journals accessible to all and disseminate our results at international scientific conferences. We will collaborate extensively with other laboratories around the world to maximise the impact of our work. We will make our research accessible to the general public, with the help of our public engagement team.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice, as they are the best understood and most widely used mammalian laboratory animal, with excellent means for generating and interpreting the effects of genetic modifications. Most of the mice will be used at young adult life stage, to study the development of the immune system or tumour development.

Typically, what will be done to an animal used in your project?

The majority of mice will be used for the generation and maintenance of genetically modified strains or for the collection of cells and tissues for analysis after the animals are humanely killed. Some mice will be injected with agents to induce superovulation. Some mice will be given single injections of tumour cells resulting in tumours under their skin. To modify gene expression some animals will be administered gene inducing agents such as doxycycline, either by injection or in their drinking water. The tumours will be monitored for size and mice humanely killed once the tumours reach a pre- determined size. Some animals with tumours may be given pharmacological or therapeutic agents to affect tumour growth. Tumour studies typically last 2-5 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?
The main adverse effect could be a mild and transient discomfort, for example following injection of tumour cells or following surgery to transfer embryos. Tumour bearing mice should not experience clinical signs other than tumour growth under the skin. Should a

palpable tumour not grow within 4 weeks, mice will be killed and excluded from the study. Repeated dosing, orally or by injection, might rarley lead to stress (due to increased handling) or irritation at the injection site. This might be expected to impact <10 mice overall. We will prioritise the least invasive methods. Adverse effects, such as pain, can affect animals undergoing surgery (e.g. embryo transfer/vasectomy), however, analgesics will be administered in consultation with the facility vet. Injections for superovulation could cause transient distress but should not cause lasting harm. All animals will be killed by a quick and humane method at the end of experiments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of mice will experience sub-threshold or mild severities at worst from breeding procedures and will be maintained in our excellent animal facility. Mice are not expected to show any overt clinical signs due to their genetic modifications. The surgical transfer of embryos to surrogate mice (used to generate new genetic strains) may cause short-lived post-operative pain and discomfort categorised as moderate severity. However, non-surgical transfer methods will be used wherever possible. Tumour-bearing mice may experience moderate severity (<5%), if subjected to repeated (e.g.
>1 week) handling and dosing of pharmacological agents (e.g. twice daily).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Physiological context is important in our research, because we study biological processes that affect tissues and the immune system, as well as cancer. The complexity of these processes cannot be adequately modelled by other means such as tissue culture, purified proteins or computational methods. Thus, we need to use animals, and in order for our data to be relevant to human biology, we need to use mammals rather than non-mammalian species in which these processes are very different. The mouse is the mammalian species most widely used, most amenable to genetic modification and best understood for such research. Thus, we must use tissues or primary cells isolated from mice and research involving experimentation in live mice, such as tumour growth, to achieve the aims of our project.

Which non-animal alternatives did you consider for use in this project?

We use cell lines (2D and 3D cultures)ultures), organoids, and purified proteins widely and wherever possible to study the protein tyrosine phosphatase signalling network and redox biology.

Why were they not suitable?

Tissue culture and purified proteins are very valuable in some aspects of our research. However, there are limits, as some cell types, e.g. from the immune system including T cells, cannot be cultured for long periods, and their protein composition cannot be modified other than by genetic means. Furthermore, tissues and tissue culture experience very different environments, therefore monitoring changes in the cell environment will be more physiologically relevant in tissues or primary cells. To understand this context, experiments on live mice, or using cells and tissues from mice, are the only options. Furthermore, we have found significant differences in the signalling in the same cells in tissue culture compared to grown as 3D tumours. The 3D environment much more closely resembles patient tumours.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have consulted with our Biostatistician, and have built on our previous experience of working with mice to ensure we use the optimal number of animals in order to make reliable scientific conclusions and avoid the unnecessary use of animals beyond those needed. We estimate that for each breeding pair, we will generate an average of 4 offspring (half of females will fall pregnant with an average litter of 8), and use mendelian frequencies of genotypes to calculate the number of pairs to set up for a particular experiment or colony maintenance. Furthermore, we have used pilot studies to help determine the size of experimental groups for tumour studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3R's Experimental Design Assistant to ensure we are considering all relevant aspects of design.

Our work is complemented by tissue culture systems, which can involve using cells from a mouse and growing more cells for our analysis, or using cell lines that are already established. We are also exploiting technical improvements to characterise the immune cells of our animals. This means we can obtain more information from a single animal, reducing the overall number of animals required. For our work with the redox biosensor strain, we plan to harvest multiple tissues/cell types using a specialised preservation technique. This means we will increase the amount of data we can derive from each animal, further reducing the overall number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to use efficient breeding to ensure we do not over-produce mice. In addition to pilot studies and harvesting multiple tissues from mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Other than for tumour studies, mice will be used for isolation of primary cells and tissues after they have been humanely killed, minimising the pain and distress of animals. The minimum number of animals will always be used that yield meaningful results, and with the lowest possible relevant severity procedure to address a specific question.

We will work in cell lines to evaluate some effects of genetic modifications prior to generating new mouse strains. In new genetically modified mouse strains, we will undertake pilot studies and test isolated primary cells to investigate effects prior to using experimental models that rely on live animals.

Why can’t you use animals that are less sentient?

The mouse is the best model organism to address our aim and objectives, as its physiology and disease processes are sufficiently similar to humans to allow us to draw meaningful conclusions, and because a wide knowledge base and many genetically modified strains and protocols exist that allow comparisons of results between projects and research groups.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our routine refinement methods include added enrichment for breeders, alternative bedding for animals with reduced motility, using established social groups where possible, habituation to handling, and providing food in gel format, additional warmth and more frequent monitoring for mice at increased risk. For established strains, we only take samples from mice from the first litter for genotyping, and we use ear rather than tail samples. We may introduce further refinement methods to protocols or husbandry methods in consultation with animal technicians and veterinary staff.

Stress and suffering of mice undergoing procedures will be minimised by observation and adherence to clear guidelines on clinical signs that trigger the end of an experiment. In rare cases where it will be required that we induce and maintain general anaesthesia, we will use modern anaesthetics and continuous monitoring.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow PREPARE and internal guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through our AWERB liaisons, who keep us informed of NC3Rs seminars and events.

139. Understanding and targeting the inflammatory response

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Inflammation, Immunology, Cytokines, Inflammatory disease, Co-morbidity

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how inflammation starts and develops and inform new treatments to limit 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?

Inflammation is a process by which your body’s immune cells and the things they make protect you from infection and tissue injury. However, too much inflammation or inflammation that last too long (referred to as ‘inappropriate’ inflammation) contributes to the development of some of the most common and devastating diseases in society, such

as Alzheimer's disease, stroke, heart disease, type II diabetes, and occurs in response to infection (such as COVID-19). Some groups of people (e.g. those with other conditions/co-morbidities such as obesity) are more likely to have 'inappropriate' inflammation that can lead to a greater severity of disease and an increased chance of mortality (e.g. seen in obese people with COVID-19), but it is not known why. Targeting 'inappropriate' inflammation with anti-inflammatories (drugs that reduce the damaging inflammation) is commonly used to treat inflammatory disease, but is often insufficient since our understanding of the how inflammatory responses start and develop is incomplete. A more detailed understanding of the inflammatory response is therefore necessary for the identification and development of future effective therapies.

Our work focuses on identifying the fundamental biological processes behind inflammation, providing a greater understanding of their impact in the development of inflammatory disease and revealing new ways to treat these diseases. This work will also aim to understand the impact co-morbidities (i.e. obesity) have in the initiation and development of inflammation, leading to the identification of tailored and more effective therapeutic strategies.

What outputs do you think you will see at the end of this project?

We will achieve a greater understanding of the biological events that promote and sustain inflammation through the identification and characterisation of critical biological mechanisms. We aim to identify signatures (such as blood markers) associated with sustained inflammation and, determine if these are altered when other diseases are present (such as obesity), directing research into the diagnosis of inflammatory disease and the development of strategies to limit inflammation. Our findings will be widely disseminated through publishing scientific papers and participation in conferences. We will make our data available to others to use in their studies. In the long term we hope that our research could provide the basis for new clinical trials to target unwanted/inappropriate inflammation in patients.

Who or what will benefit from these outputs, and how?

The main benefits of this research will be the generation of new knowledge on how inflammation starts and how it can become uncontrolled leading to the development of disease. Since inflammation underpins the development of multiple diseases, our research will benefit preclinical and clinical research on a range of disease disciplines. We aim to identify new biological processes and biomarkers fundamental to inflammation assisting with basic and clinical research into therapeutic approaches to target inflammatory disease. In the short term, our research will benefit other researchers, the pharmaceutical industry, and clinicians studying the development of inflammatory disease. In the long term, our research will help guide the development of future diagnostic tools and therapies to benefit patients that suffer from diseases driven by inappropriate inflammation.

How will you look to maximise the outputs of this work?

We will publish our findings from these studies in respected, open access journals, present our data at leading international conferences, and utilise pre-print servers to maximise the dissemination of our research. We collaborate with other universities, to assist with identification and development/repurposing of effective anti-inflammatories.

Species and numbers of animals expected to be used

- Mice: 1600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult mice in our studies. This includes normal control and sometimes genetically altered mice or those that are obese. The immune system of a mouse is similar to that of a human and it responds in a comparable way to infection, injury, and in disease, and mice are therefore appropriate for this work. Infection, injury and disease can occur at any age, so we will use adult mice to represent the largest percentage of the population.

Typically, what will be done to an animal used in your project?

The main procedure in this project involves the induction of inflammation in mice using established stimuli/agents by giving injections of substances that mimic an infection. These agents will be delivered either through injection (subcutaneously, intraperitoneally or intravenously), orally (oral gavage) or via nasal inhalation. In a typical experiment, these treatments may cause a fever or some sickness-like behaviour in the animals, but this will be monitored closely and kept to a minimum by using the lowest exposures/doses possible. In our experience, mice do tolerate inflammatory agents well and sickness- like behaviour is often short lived. Animals will be humanely killed within a few hours after the injections and tissues taken to assess the inflammatory response. Sometimes we will also use agents to try to inhibit the inflammatory response, which will mean the animals should feel much better. In some experiments we will keep the mice for a bit longer (usually up to 10 days) to give the inflammatory response more time to develop. In a few experiments we might also measure how much food the mice eat and how much they weigh. Food intake and body weight measurements are not invasive or disruptive to the animals and are often monitored to assess well-being. In some circumstances, we will monitor body temperature with a rectal probe as part of monitoring the welfare of the mice. For some experiments we might also use mice that have obesity as this condition has been shown to affect how people respond to infection, injury and disease. Most often, we will make the mice obese by feeding them a diet high in calories, usually one containing a lot of fat as this is how most humans become obese. Sometimes we will use mice that have altered genes, which make them eat a lot and become very overweight. In addition to these procedures, mice will be used in this project for breeding, and for collection of cells and tissues for *in vitro* laboratory-based studies. At the end of all experiments, animals will be humanely killed, and blood and tissues taken to assess the inflammatory response in more detail.

What are the expected impacts and/or adverse effects for the animals during your project?

We will cause an inflammatory reaction in the animals by injecting them with agents that mimic an infection. The animals will experience some sickness and the impact will be similar to what humans experience when sick with an infection and symptoms could include lethargy, fever and reduced appetite. However, the symptoms will usually only last for a few hours before the animals are humanely killed to take tissues for analysis. In those tissues we will then look at markers of inflammation, such as identification of immune cells and the agents that they release. In the experiments where we keep animals for longer the

mice might also have some sickness behaviour but this will tend to be milder. The mice that we use for breeding will not experience any adverse effects.

Feeding mice a high-fat diet leads to weight gain and obesity. Over time, obesity may lead to insulin resistance and low-grade inflammation (similar to that observed in individuals with type 2 diabetes). Together these effects of obesity may cause subtle changes in behaviour (e.g. reduced activity), but are not associated with any lasting suffering or distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold/Mild (30%)

Moderate (70%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Inflammation is a complex response that involves many different tissues and organs that cannot be modelled accurately in *in vitro* systems. An inflammatory response also produces symptoms such as reduced appetite, which is due to an interaction of the immune system with the nervous system and the brain. Thus, the whole body is involved in an inflammatory response and as such whole animals are needed to understand this complexity. The proposed studies could also not be undertaken in lower species because they do not show such similarities to humans including their immune system.

Which non-animal alternatives did you consider for use in this project?

We did consider lower species (e.g. zebra fish at an age before protection) and where appropriate these will be used. The wax moth larvae (Galleria mellonella) was also considered as an infection model, but it lacks some of the key features of the human immune system. We are routinely using *in vitro* models and data from these experiments will help us design our experiments using animals. Many of our *in vivo* studies will use a partial replacement by performing *in vitro* experiments in parallel, reducing the numbers of animals needed.

Why were they not suitable?

These lower species, such as zebrafish, do not always show such similarities to humans including their immune system. Of relevance to our project, they do not have some of the

important molecules that we are interested in, such as mammalian-specific inflammatory mediators (e.g. interleukin-1 alpha; IL-1α), and other inflammatory pathways are known to function differently in lower species (e.g. the inflammasomes). These molecules are implicated with human disease, and so understanding their contribution to inflammation is an important feature of our models. *In vitro* studies do not fully mimic the complexities of the multiple tissue systems and organs involved in an inflammatory response to infections/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 have used our previous experience of performing experiments of this type to help us decide the best way to answer the questions we are asking while using the minimum number of animals. For example, estimates of animal use is based on i) previous work and experience using the relevant methodologies, the parameters to be studied, and specific mouse models; ii) the scope and objectives of the current project; and iii) careful consideration of experimental design.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Several factors lead to a reduction of animal numbers, including reducing variation (e.g. keeping the environment consistent), good experimental design (including the use of the NC3R's Experimental Design Assistant) and the use of appropriate statistics. In particular, statistical tests will be used to ensure that we use the minimum number of animals possible to reliably interpret our data and also so we can refine our questions to then design the most informative experiments. Whenever we get new data, we will always re-do our calculations in order to make sure we are still using an appropriate animal number to achieve our aims.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will minimise the number of animals bred by using efficient breeding strategies and by using mice of both sexes whenever possible, but for many studies we will obtain animals from commercial breeders. We usually take several tissues from the animals at the end of the experiments for multiple analyses (and sharing of tissue), which often leads to additional scientific questions. Whenever we perform analyses that leads to a large quantity of data (such as RNA-seq to analyse all genes) we will make our data freely available to other groups so they can analyse it to answer their own research questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Species: We will use mice as there are no *in vitro* alternatives that can fully replicate the inflammatory response after infection, tissue injury and disease, and rodents are the lowest species that have most of the important components of inflammation (e.g. IL-1α and inflammasomes).

Models: In some experiments we will also use obese mice as a model of co-morbidity that alters how the immune system responds to infection, injury and disease. Mice will usually be made obese by access to a high-fat diet. Alternatively, we might use mice that have altered genes that make them obese. These models, therefore, do not involve any surgery or painful procedures to induce the obesity.

A key objective of this project licence is to identify the biological events underlying systemic inflammation induced by infection or tissue injury. In some experiments, we aim to reduce inflammation by giving appropriate inhibitors/modulators so the response should be less in these animals.

Measurements: For a lot of studies animals will be humanely killed only after a few hours after induction of inflammation (e.g. 2h). This early time point is a result of a refinement in which for some of our questions we can now assess some important/key mediators/parameters after only 2h. For longer term studies (e.g. up to 10 days) animals will be monitored over time for well-being.

In some studies, we will require a cardiac blood sample followed by perfusion with fixative in order to best preserve the tissue for subsequent analysis. In this situation this will be done under terminal anaesthesia.

Why can’t you use animals that are less sentient?

Our objectives cannot be fully achieved using less sentient animals (such as fish/insects) or with very young (neonate) mice mainly due to differences in their immune system, and adult mice are therefore needed. However, we will use zebrafish whenever possible and perform *in vitro* studies in immune cells before we move to mice. However, zebrafish cannot be used for all studies as they do not have exactly the same immune system as humans (and mice) and the way in which their cells produce some inflammatory molecules can be different. As some of our studies involve keeping animals for up to 10 days terminal anaesthesia cannot be used. For some of the acute studies where mice are humanely killed after 2-6h induction of inflammation, if a second injection/stimulus is required (e.g ATP, a marker of tissue damage) this will be done under terminal anaesthesia as mice are usually humanely killed 15 mins later.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In a significant amount studies, animals will be humanely killed within 2-6h with minimal adverse effects on the mice. We have further refined some protocols and mice can now be

humanely killed at a 2h time point in some studies. We need to keep some animals for longer (up to 24h) as some of our primary outcome measures (e.g. in the blood) will not be elevated as early as 2h. However, we will continue to develop our methods of *ex vivo* analysis and if further outcome measures become apparent, we will refine methods to also allow mice to be humanely killed earlier.

For studies where we want to study sustained inflammation, we will keep animals for longer (e.g. 10 days). However, we will continually assess which are the best outcome measures and where appropriate animals might be humanely killed earlier. In these studies mice will be monitored frequently.

For all studies and at all times, mice will be handled appropriately by trained researchers (e.g. using tube handling for movement in and out of cages) and the use of suitable home cage enrichment.

As there is no surgery in our experiments, post-operative care and pain management is not applicable.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date with literature, publications and recommendations from the most appropriate bodies such as the NC3Rs and LASA, as well being informed from communication with the NVS and NIO and developments within the scientific community in general. For example, for refinements involving injections we refer to Morton et al 2001 and https://researchanimaltraining.com/articles/an- introduction-to-the-administration-of-substances/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will at all times aim to implement any advances in techniques that adhere to the 3Rs and improve the welfare of the animals. We will stay up to date with the NC3Rs literature and recommendations as well being informed from communication with the NVS and NIO and the scientific literature in general.

140. Drug Metabolism, Pharmacokinetics and PK/PD of Substances for Drug Discovery 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

Key words
pharmacokinetics, pharmacokinetic/pharmacodynamic relationship, concentration-time profile

Animal types	Life stages
Mice	adult
Rats	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary aim of this project is to evaluate what the body does to the substance (pharmacokinetics) and whether the substance has the required effect in the body (Pharmacodynamics).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 will focus on developing new medicines for a range of diseases, including immuno- inflammation, respiratory, oncology and pain. Such diseases affect millions of people who are in need of improved, effective treatments to either cure the disease or improve their quality of life.
Substances will be evaluated as they are discovered and developed as potential new medicines. They will be assessed in animals (mice, rats, rabbits) to predict what the

human body does to the substance (pharmacokinetics) and whether the substance has the required effect in the human body (pharmacodynamics). The analysis of the concentrations in the blood and tissues of animals following administration helps us to understand how much of these potential new medicines will be required to be administered to humans to be both effective and safe.

What outputs do you think you will see at the end of this project?

The amount of test substance in the blood over time (the concentration time profile) allows us to determine the rate and extent of absorption from the site of substance administration, distribution throughout the body and the rate of removal and excretion from the body. Selection of substances for further evaluation is based on the potential to reach the target organ/site of action and be retained for sufficient time and at a concentration appropriate for assessment in a subsequent pharmacology (efficacy) study.

The concentration time profile generated in animals is used to predict what will happen to the substance in the human body (absorption, distribution, metabolism and excretion). This type of data, generated early in the research program enables us to mitigate the risk of progressing substances as potential medicines that the body handles poorly, through rapid metabolism or poor absorption. Alongside the animal (pre-clinical) pharmacology data, the pharmacokinetic data we obtain allows us to predict the likely human dose and frequency of dosing required in the clinic.

Who or what will benefit from these outputs, and how?

Pharmacokinetic evaluation of new substances enables a greater understanding of the link between blood and/or plasma concentrations and the effects of these substances in a range of animal models. Subsequent studies benefit from this prior understanding of the concentration time profile through accuracy in dose and formulation prediction and the suitability of substances put forward into additional studies. This reduces the number of animal studies performed at unsuitable doses or with sub-optimal substances. In addition the identification of an appropriate animal species in which new substances have the appropriate exposure will help assist in their safety (toxicological) evaluation and could reduce animal testing with unsuitable dose formulations. Pharmacokinetic data generated in the animal (pre-clinical species) forms part of regulatory submission which is required for progressing substances into evaluation in humans (clinical). These benefits will be ongoing throughtout the lifespan of the Project Licence.

The ultimate benefit will be to the waiting patients. The pharmacokinetic evaluation of potential drug candidates is an important piece of the jigsaw that leads to the discovery, development and marketing of new therapeutic agents for the treatment or prevention of diseases in man. This benefit will be ongoing throughout the lifespan of the Project Licence.

How will you look to maximise the outputs of this work?

Physiologically based pharmacokinetic computer models are routinely built and refined using the information obtained from pharmacokinetic studies for a number of Projects which can lead to a substantial reduction in the need for additional animal experiments. Study data and knowledge gained will be shared within the company and with collaborators and applied to benefit future work. Data and knowledge will also be shared outside the company in the form of posters at scientific conferences and publications in scientific journals

Species and numbers of animals expected to be used

- Mice: 7550
- Rats: 9800
- Rabbits: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice, rats and rabbits will be routinely used on this project since they have developed physiology and organ systems similar enough to humans to perform studies in these species that enable prediction of what will happen to the substance when it is administered to humans in a clinical trial or a patient. In addition adult mice, rats and rabbits align with the ages of animals used in the disease (efficacy) model and non-clinical safety studies. For Regulatory Authorities to give permission to conduct human trials of potential new medicines they require a prediction of dose, concentration and safety profile in humans. Studies conducted under this project are designed to progress substances, with the most appropriate concentration profile, through the development process and enable future characterisation of efficacy and/or toxicity.

Mice and rats are acceptable to Regulatory Authorities for the prediction of human pharmacokinetics, provide reliable pharmacological models to test the effectiveness of substances and are the principal rodent species of choice in safety testing. In certain circumstances the rabbit may be the species of choice (e.g. reproductive toxicity) and therefore an understanding of the pharmacokinetics in this species will be required to aid the study design.

Typically, what will be done to an animal used in your project?

Protocol 1 (Pharmacokinetics): In some cases animals may receive (typically on a single occasion) a substance to alter the physiology prior to substance administration. However, as routine, healthy animals will have substances administered by various routes but typically orally. This could be as either a single or repeat administration. Blood samples will be taken either via a needle or cannula inserted into a blood vessel near the surface of the body (i.e. tail vein for rat or ear for rabbit) or directly via a small puncture wound over the tail vein (rat or mouse) in order to determine the concentration of a substance over a time course. At the end of the study animals will be humanely killed and tissues may be collected to determine the substance concentration in those tissues. Where appropriate the effect of any given substance on the pharmacological response produced will be assessed by measuring substance concentrations and/or markers of treatment effects (i.e. cytokines) in the blood. Animals will be weighed and general health assessed daily throughout the study duration. The duration of an experiment is determined by the type of substance being administered (short or long acting), the frequency of dosing (single or multiple doses) and the number of phases to an experiment (if performing comparative formulations, doses or routes within the same animals).

OR

Protocol 2 (Surgical Cannulation in Rodent with Recovery): Rats and mice will be surgically prepared (under general anaesthesia) with a maximum of 2 implanted venous cannula(e). In addition a laparotomy may be performed and up to 3 cannulae inserted (for alternate dosing routes or sampling of biofluids). A vascular access button may be attached to the venous cannula(e) or the animal placed in a suitable device (e.g. jacket and tether system) to protect the indwelling cannula(e). Peri-operative analgesia is administered to minimise any pain, suffering and distress associated with the surgical procedures. The rats and mice will typically have a minimum of 72h recovery prior to continued use on Protocol 3 or 4.

AND

Protocol 3 (Pharmacokinetics in Surgically Prepared Rodents): Following recovery from surgery, in some cases animals may receive (typically on a single occasion) a substance to alter the physiology (e.g. transporter inhibitor) or a pharmacological tool (e.g. an inflammatory agent) prior to substance administration. However, as routine, healthy animals will have substance(s) administered by various routes but typically intravenously as an infusion. This could be either a single (short duration e.g 1 h or sustained e.g. 48h) or repeat administration. Blood samples will be taken via cannula which has been surgically implanted into a vein in order to determine concentration of a substance over a time course. The concentration of the substance in blood (or plasma/tissue) will be determined. Where appropriate the effect of any given substance on the pharmacological response produced will be assessed by measuring substance levels and/or markers of treatment effects (i.e. cytokines) in the blood. Animals will be weighed and general health assessed daily throughout the study duration. The duration of an experiment is determined by the type of substance being administered (short or long acting), the frequency of dosing (single or multiple doses) and the number of phases to an experiment (if performing comparative formulations, doses or routes within the same animals). At the end of the study animals may be humanely killed and further blood and/or tissues may be collected for additional analysis.

However, if the animals are determined by the Named Veterinary Surgeon to be fit to be kept alive for re-use, they will be maintained under the protocol to undergo further dosing/sampling cycles to investigate the pharmacokinetics of additional substances of interest. Animals may be transferred to protocol 4 for reuse.

OR

Protocol 4 (Pharmacokinetics in Surgically Prepared Rodents Non Recovery): In some cases, prior to surgery, animals may receive (typically on a single occasion) a substance to alter the physiology (e.g. transporter inhibitor) or a pharmacological tool (e.g. an inflammatory agent) prior to substance administration. The animals may undergo surgical cannulation of organs, the appropriate blood vessels and the organs perfused during the experiment. Following surgery the animals will remain under terminal anaesthesia and will have substance(s) administered by various routes but typically as an infusion into a blood vessel. This could be either a short duration e.g administered over less than 5 minutes or sustained infusion e.g. 1 - 7h). Biofluid (e.g. blood, bile, urine) samples will be taken via cannula which has been surgically implanted to determine concentration of a substance over a time course. At the end of the study animals will be humanely killed and further blood and/or tissues may be collected for additional analysis. Animals may occasionally be transferred onto this protocol, from protocol 3.

OR

Protocol 5 (Control Biofluid and/or Tissue Collection): Blood samples may be removed from a superficial vein on a single or serial occasions. Animals may be housed in a metabolism cage (up to 24h duration) to enable collection of control urine/faeces. Where large volume of blood or control organs are required the animal will undergo terminal anaesthesia and the tissues, organs and/or biofluids collected. At the end of the study animals will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse events are not expected with dosing and blood sampling due to the low doses typically administered under this project and refined techniques of blood sampling. Non-specific signs rarely observed include weight loss (on repeat substance administration) discharge from eyes and/or nose, altered coat condition, reduced activity, behavioral or postural changes, reduction in food and water intake, abnormal faeces, abnormal breathing, fur staining, alteration of body temperature, irritation/discoloration/crusts/ulceration of the skin and swelling or masses under the skin. Any adverse events are likely to be transient in nature, with clear signs of improvement within 2 hours of the onset of signs, and throughout the day following dosing. Animals will be monitored and undergo regular observation and action will be taken to minimise adverse effects. This may result in animals being killed to prevent further suffering.

During surgery there is a risk that a blood vessel may snap/shred or display bifurcation (branching) and therefore unsuitable to insert a cannula. In these circumstances the animal will be terminated and used for control tissues/biofluids. In addition there is a risk that the animal may stop breathing under anaesthesia. Post surgery a small amount of temporary weight loss can occur whilst other post operative complications are rare e.g. suture breakage, dryness/dead skin or infection at the surgical site. The vascular button system or harness restrain system may result in mild friction injuries, irritation, inflammation or infection to the area of contact. The development of swellings around the button site may occur however this will have negligible impact on the animals welfare. On rare occasions the presence of indwelling cannula in a blood vessel may lead to unexpected thrombosis, inflammation of the vessel and infection (swelling, redness or discharge at site of wound).

During procedures such as substance administration or blood sampling animals are restrained to avoid injury to themselves and ensure success of procedures. There is a low risk of misdosing or cannula being dislodged. Patency of cannula(e) is maintained by flushing with suitable solution in an aseptic manner. There is a small risk that this flushing procedure may dislodge a clot resulting in the potential death of the animal. This is minimised by regular (weekly) flushing and minimal applied pressure should there be any resistance.

For some studies animals are housed on their own to either avoid post operative complications, if dosed with different substances, if administered at different doses or to collect urine and faeces for substance analysis. Animals are monitored for general signs of stress or discomfort during this time.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Experience under previous PK and PK/PD Projects indicates that at least 55% of animals are expected to show no more than mild effects following treatment. Up to 30% of animals will likely, experience a cumulative moderate severity, or at least a period of moderate severity at some point during experiments.

Protocol 1 is moderate however approximately 70% of rats and mice are expected to experience mild severity, approximately 30% of rats and mice will experience moderate severity. Approximately 100% of rabbits are expected to experience mild severity.

Protocol 2 is moderate. All animals will be subjected to surgical procedures, 100% of rats and mice are expected to experience moderate severity .

Protocol 3 is moderate however approximately 70% of rats and mice are expected to experience mild severity, approximately 30% of rats and mice will experience moderate severity.

Protocol 4 is mild with approximately 30% of rats and mice expected to experience mild severity when dosed prior to terminal anaesthesia with 100% of rats and mice progressing to non-recovery severity.

Protocol 5 is mild with approximately 20% of rats, mice and rabbits expected to experience mild severity when sampled for serial control matrix collection with approximately 80% of rats, mice and rabbits to be non-recovery severity as control matrix collected under terminal anaesthesia.

Approximately 5% of the total number of rat and mouse studies conducted under this project are anticipated to be Pharmacological efficacy studies.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?
Preliminary triaging utilising in silico, in vitro and modeling techniques will occur reducing the number of substances from thousands to hundreds, to finally only those demonstrating potential as a new medicine progressing to investigation in animals. These substances need to be tested in animals due to the complexity of the mammalian body and the different interactions between the body and new medicines. Understanding these interactions and hence understanding the concentrations of a substance following administration via the desired route of dosing cannot be achieved without using the 'whole body system' of animals. Without this information the risk of ineffectiveness of a new medicines against a disease or it's potential toxicity would be too high to give to humans.

Which non-animal alternatives did you consider for use in this project?

Whilst in silico, in vitro and modeling methodology is employed for triaging compounds to select those with greatest potential to deliver a suitable clinical profile (i.e. balance between efficacy and concentration) the models are limited as they do not represent an integrated whole body system. There has been much progress with the development of non-animal alternatives such as Quantitative Structure-Activity Relationship (QSAR) models (Review on QSAR modeling. J Anal Pharm Res. 2018;7(2)), they are not yet at a stage where the relevance of the data generated from them for humans is fully understood and accepted as suitable alternatives by Regulatory Authorities. Similarly, experiments using many small pieces of isolated tissues or cells do not yet reproduce the level of complexity and integration of body systems within a living animal such as the ability of cells and organs to continuously communicate. Non animal alternatives are also not yet adequately able to simulate absorption, distribution, metabolism and elimination of medicines throughout the body. Consequently, non-animal alternatives are not yet fully characterised and/or validated to provide confidence to replace data from studies in animals (Ref: Indian J Pharm Sci. 2011 Jan-Feb; 73(1): 1–6; J Pharmacol Toxicol Methods. May-Jun 2013;67(3):203-13).

The company continues to seek alternatives to animal use in research and development to reduce the number of studies requiring the use of animals. However any alternatives must be shown to be reliable, robust and acceptable by government organisations that regulate new medicines for patient use globally.

Why were they not suitable?

In order to generate relevant decision making information from studies conducted under this project they need to use the same experimental animal models to correlate with those studies required by Regulatory Authorities before a potential new medicine can be given to humans. There are currently no non-animal models that are considered acceptable to drug regulatory authorities for this purpose and it is therefore not possible to consider the use of non animal alternatives for this project at this time (J Pharm Pharm Sci 24, 113 - 126, 2021).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 similar work and projects previously supported under licence authority within the company. 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 of those readouts to inform on animal numbers required per type of study to produce statistically useful information.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whenever possible blood samples will be collected using microsampling technology (reference: nc3rs.org.uk/microsampling). In addition continuous advances in analytical detection sensitivity could enable even smaller blood sample volumes to be accurately taken from an animal and the blood concentration of substance(s) at a single time-point determined. Reducing the volume of blood required per sample enables a serial concentration-time profile to be determined in a single animal rather than using multiple animals to generate a composite profile. Therefore the use of serial blood sampling reduces the number of animals required to generate a pharmacokinetic profile. It could also enable the simultaneous collection of blood to measure pharmacological biomarkers and blood concentration within the same animal. This may lead to a more rapid decision on a series of substances, eliminate the need for additional studies to be performed at a later date or enable termination of a substance earlier in the drug discovery process and a downstream reduction in animal usage.

A pool of scientists are available to provide expertise to assist with the design, conduct and interpretation of the studies. Internal scientific and ethical review of studies ensures that the number of animal studies are minimised. Statistics and study design are optimised to ensure the minimum numbers of animals are used to achieve the aims of the study and project and advice is available and taken from a qualified in-house statistician who provides dedicated support for the project. The use of robust study design principles will be used to maximise the likelihood of generating non-biased experimental results and limit the number of animals needed to generate good quality decision making data.

When investigating the concentration of a substance following administration via different routes, formulation and/or doses the same animal may be used. In doing so the comparisons are made intra rather than inter animal, thereby reducing variability and animal numbers.

Where possible in vitro based assays will be planned so that minimal amount of control tissues are required and, where appropriate, samples of control tissues or body fluids will be collected for use in future investigations to reduce animal use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Surgically prepared animals typically experience a higher degree of suffering as a result of surgical procedures than that experienced during the course of the rest of the study. Therefore re-use of surgically prepared animals for more than one study significantly reduces the number of animals undergoing a higher degree of suffering. Wherever possible re-use of both surgical and non-surgically prepared animals may be undertaken, under veterinary guidance, where this is considered to be of lower impact than using another animal.

To help minimise experimental variation, studies are conducted in facilities providing an optimal environment suitable for the species, with a limited number of dedicated staff performing the technical procedures, animal care and husbandry. This will help to ensure

that animal group sizes are the smallest possible to achieve the scientific objectives of the study.

Studies conducted at higher doses than typically used in this project may involve ‘staggered starts’ to limit the risk of adverse effects to the animals. Existing blood concentration and in vitro potency data will also be reviewed from similar substances before choosing doses.

Animals will be randomly assigned to experimental groups using a computer-generated random number generation system which will reduce bias in animal allocation that could influence the data generated.

Where appropriate, samples of control tissues or body fluids will be collected for use in future investigations in order to reduce animal use.

Although experimental work authorised by this project is not performed using Good Laboratory Practice (GLP) guidelines it is performed to the general standards of GLP to ensure quality and data integrity.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The work conducted under this Project Licence will evaluate substances as they are discovered and developed, have undergone in silico and in vitro analysis and are deemed of further interest. To help understand how much of these potential new medicines will need to be given to humans to be effective and safe the concentrations in blood, biofluids and/or tissues following administration via an appropriate route of delivery will first need to be investigated in animals.

The doses administered to determine blood, biofluid and/or tissue concentrations are chosen such that the exposure achieved in the animal will have minimal risk of adverse events. The dose route and volumes of administration and the number of blood samples taken will be kept to a minimum to complete the scientific study objectives, whilst aiming to ensure the welfare of the animals is not compromised and that the level of discomfort is kept to a minimum. Non surgically prepared animals will be warmed prior to blood sampling to facilitate successful blood collection. This will be kept to the shortest time possible with an aim to give the smallest impact to the animal. The use of microsampling helps to achieve this aim. Using the tail nick method for blood collection in mice, in conjunction with microsampling, allows for fewer procedures to be carried out; for example, fewer times in a warming cabinet, and fewer punctures are required for blood collection as the fresh scab is gently removed to allow serial bleeds to be carried out from one puncture over a short period of time.

Surgically prepared animal models are used to enable substance to be administered by intravenous infusion to assess the pharmacokinetic profile. In addition serial blood sampling via an indwelling cannula reduces the need for handling and manipulation of the animal thereby reducing any distress and allowing the animal to continue to exhibit normal behaviours during the procedure. Peri-operative care e.g. warm environment, supplementary fluids, supportive food, analgesia, aseptic technique and antibiotics (if required) all contribute to minimise the potential of surgery to have pain, suffering, distress and lasting harm on the animals. The use of vascular access button system to protect the rodent surgically implanted cannula will minimise the need for individual housing of cannulated rats. Duration of surgery, dosing and sampling is kept to a minimum required for individual studies to minimise their impact on the animals whilst ensuring quality decision-making data is obtained. Exploration of the use of vascular access buttons in group housed female mice may minimise the need for individual housing in cannulated mice.

The core study designs are based on internal guidance in order to provide quality decision-making data balancing the need to achieve study objectives while minimising animal use. The core study designs have been used extensively under previous project licences, and in other facilities, and have proved successful in characterising and/or assessing the concentrations of test substances in the body. They are generally in line with those used throughout the pharmaceutical industry.

The combination of activities outlined in this section will ensue that the study objectives are achieved whilst causing the least pain, suffering, distress, or lasting harm to the animals.

Why can’t you use animals that are less sentient?

Rodents are a relevant species as they provide many similarities with the mechanisms, systems and processes involved in human absorption, distribution, metabolism and excretion of a potential new medicine that are not known to be fully active in less sentient species. In addition there is a regulatory requirement to characterise the concentration-time profile of potential new medicines in mammalian species (rodents and non rodents) during the programme of regulatory studies. Preliminary pharmacokinetic studies need to use the same species intended for the efficacy, safety and regulatory programme and accepted by Regulatory Authorities. This is to ensure the outcome of the preliminary studies are likely to be predictive of the outcome of the studies conducted during the regulatory programme. Since findings in animal studies are used to understand the potential concentration, efficacy and safety risk when giving a potential new medicine to humans the experiments conducted under this project will normally be conducted in conscious young adult animals with complex, integrated and well-developed physiological mechanisms and organ systems representing those of patients. Due to the duration of the majority of the models to be used, it is impractical and unethical to keep animals anaesthetised for the duration of the required procedures and sample collection periods.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experiments described within this project will undergo continual review to ensure that the number and frequency of procedures and necessary harms caused will be kept to the minimum required to achieve the study objective.

Warming cabinets will be used for post-surgery recovery and wet food/mash will be provided to ensure all animals recover as quickly as possible. All animals will be monitored

closely following surgery, dosing and sampling. Where the condition of an animal gives cause for concern, observations will be continued until clear signs of recovery are evident or further action will be taken to ameliorate them.
Additional refinements to the cage environment (e.g. provision of enrichment) and good practice in animal handling will continue to be considered and implemented where practicable throughout the duration of this licence.

During the course of the previous licence, various refinements were implemented including:

Use of "button system" to protect rodent surgically implanted cannula(e) as a replacement to the "harness and tether system". This minimises the time spent singly housed as well as improving body weight gain post surgery.

Use of pulseoximeter for anaesthetic monitoring of rodents during surgical procedures.

Use of nesting material to provide environmental enrichment for rats without compromising regulatory requirements.

Use of additional nesting material as well as paper based nesting material to provide environmental enrichment to mice without compromising regulatory requirements.

Changing the way that mice are restrained for procedures by using a soft (e.g. sponge) rather than a rigid surface.

Handling of mice using tube/tunnel/cupping method.

Where possible the use of an in-house mouse restrainer that enables the mouse to be restrained by the tail but with free body movement.

Acclimatisation of animals to handling for procedures likely to be encountered on study prior to surgery being implemented.

Regular attendance at the scientific and ethical review forum by representatives from research statistics as part of the scientific and ethical review of studies.

Where possible the introduction of enhanced, multilevel caging for group housed rodents. Group housing of rabbits in floor pens in preference to caging to allow habituation and handling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The following published documents will advise on experimental design, animal welfare and husbandry during the life cycle of this licence:

NC3Rs - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies (2019).

Smith A et al (2018). PREPARE: guidelines for planning animal research and testing. Lab Anim; 52(2):135-141.

Prescott MJ, Lidster K. Improving the quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46:152-156, (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 (2017)

LASA - Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017)

LASA - Guiding principles on good practice for animal welfare and ethical review bodies. (2015)

Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).

Kilkenny C et al (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6).

A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes Diehl et al, (2001), Journal of Applied Toxicology, 21, 15-23.

Morton DB et al., (1993) Removal of blood from laboratory mammals and birds Laboratory Animals 27, 1-22

Hurst, J., West, R. Taming anxiety in laboratory mice. Nat Methods 7, 825–826 (2010).

Henderson, L.J., Dani, B., Serrano, E.M.N. et al. Benefits of tunnel handling persist after repeated restraint, injection and anaesthesia. Sci Rep 10, 14562 (2020).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Company's Named Information Officer (NIO) will help distribute information associated with animal welfare, best practice and 3Rs. The project licence holder and other key personnel who operate under this licence, also regularly attend the Company's internal Animal Welfare and Ethical Review Body (AWERB) meetings, where animal welfare, best practice and 3Rs related information is shared.
Furthermore, regular referral to the NC3Rs website, published literature (including new or revised guidance issued by Regulatory Authorities), and feedback from external conferences, symposia and workshops will ensure that advances in 3Rs are identified. 3Rs matters are highlighted, discussed and action taken to implement within the Company via these forums.

141. Safety, residues and pharmacokinetics of veterinary pharmaceuticals, biologicals, feed additives, feed components and contaminants, and vaccines, including their first use in the target farm animal.

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

Safety, Vaccines, Pharmacokinetics, Additives, Residues

Animal types	Life stages
Cattle	juvenile, adult, pregnant, neonate
Sheep	adult, juvenile, neonate, pregnant
Pigs	juvenile, adult, neonate, pregnant
Domestic fowl (Gallus gallus domesticus)	juvenile, adult
Game birds	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to complete a series of individual studies to evaluate safety to the animal, residue safety for the consumer and the pharmacokinetics of existing, novel or generic veterinary medicines, biologicals, feed additives, feed components and contaminants, and vaccines. This may be for early preclinical work which may include first use in the target species or for later clinical studies including reformulation of current pharmaceuticals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within 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 conduct animal studies looking at the safety, residues and pharmacokinetics of a new or existing medicine, biological, feed additive, feed component, contaminant or vaccine before either larger studies are performed, and necessary before any application is submitted for regulatory approval. This minimises the risks to animal health and wellbeing as well as human health risks. Safety and residue tests are a crucial step for new or repurposed pharmaceutical products on their journey to being approved by the appropriate regulating authority (outlined in VICH guidelines GL43 and GL46). During a pharmacokinetic study it's important to understand the metabolism of the therapeutic dose of the product and how it interacts within the target species (Guidance will be sought from VICH GL52 and EMEA/CVMP/133/99).

What outputs do you think you will see at the end of this project?

Multiple scientific reports for the sponsoring companies that will potentially lead to new treatments for animal diseases, and conditions that cause economic and animal losses, together with animal welfare impacts, and human health each year. Our continuing input into fundamental animal health research and scientific papers published in relevant journals. We play an active role in maintaining and updating guidance on animal health and wellbeing and best practise for these types of safety studies.

Who or what will benefit from these outputs, and how?

Animals, pharmaceutical companies, farmers and the wider economy will all benefit from the research carried out. The testing facilities provided may result in new and novel products being brought to market to help alleviate many different animal diseases. Therefore, this benefits the animal's wellbeing and health, farmers see improved yields and pharmaceutical companies see profits by marketing their product. Human health benefits from access to safe food and/or veterinary interventions. These factors combined stimulate and help the wider economy to grow within the UK. We earn income that assists the maintenance and development of our facilities.

How will you look to maximise the outputs of this work?

We will work with companies, institutes and charities to disseminate the study findings, publish research papers, attend relevant conferences and we also play an active role in creating guidelines in this area of research.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 400
- Other birds: No answer provided
- Cattle: 50
- Sheep: 100
- Pigs: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Many of our studies at are conducted in the target species for authorisation and/or support of a veterinary medicine, biological, feedstuff, feed additive or vaccine and such testing is required by the regulatory authorities. There is a policy of checking whether non-animal alternatives are possible, for example by regular visits to the NC3Rs website (http://www.nc3rs.org.uk) and attending relevant conferences. Where there is a validated and appropriate in vitro model then the client would be informed and the in-vivo work refused.

Typically, what will be done to an animal used in your project?

Administration of substances either; in feed or water, orally by gavage, injection (subcutaneous, intramuscular or intravenous), intramammary (via teat canal), intranasally, topically to the skin or via a non-surgically implanted slow release device. Blood may also be taken, cannulas inserted, biopsies of superficial tissues such as hair, fleece, skin or feathers, nasopharyngeal swabbing, cloacal swabbing, faecal sampling and percutaneous secretion sampling (by needle but only once in the last 24 hours of a study) may also be performed. Lactating cows may be dried off.

What are the expected impacts and/or adverse effects for the animals during your project?

Likely adverse effects:

There may be signs of local or systemic treatment intolerance particularly where multiples of normal or proposed dose-rates are used during target animal safety studies. These include:

Local reactions either injection site reactions or site of application reactions (including signs of pain associated with the area, swelling, skin changes)

Systemic reactions to the substance (behavioural changes, mobility changes, feed intake and weight gain changes, organ system changes)

The volume of blood removed could result in anaemia or systemic signs, although this is managed by calculation and reference to guidance on suitable blood volumes that can be safely removed over time

Anaphylaxis following administration of substances Likely incidence:
The substances that have been researched before are likely to have undergone preclinical studies in, for example, rats or mice and most likely the target species already. On occasion, there may be either transient clinical signs or mild lasting clinical signs at normal dose rates, which may be accentuated in higher doses administered in tolerance/safety/residue studies. This is specific to the treatment, its regimen and formulation.

First use in the species carry additional risk as prior experience in e.g. mice, rats or in vitro may not accurately predict the effects in farm species. In addition the product may be in a preliminary formulation. This is specific to the treatment, its regimen and formulation.

Typically pharmaceutical safety studies have groups treated at 1x, 3x and 5x dose-rates and sometimes at repeated intervals (lasting up to 90 days). Depending on the product, there may be clinical signs associated with treatment at the 3x or 5x levels.

Reproductive studies are conducted at up to 3x the recommended maximum dose rate in adult animals including pregnant females. The 3x maximum dose rate could be associated with clinical signs in the adult animal or the offspring.

Vaccines may be tested at up to 10x normal dose-rate.

Repeated blood sampling could result in clinical signs after either a large volume was taken on a single occasion or repeated sampling over a period of time. This is very unlikely to occur as the guidance in the appendices is followed which includes LASA guideline for blood withdrawal.

Anaphylaxis is a rare but possible event.

Measures to prevent, recognise and control occurrence and severity:

Prior knowledge will be reviewed to ensure that doses and dose-rate multiples are unlikely to exceed moderate severity

If they are deemed likely to exceed moderate the work will be declined or the work will be discussed with the Home Office Inspectorate

The protocol will include adverse events that might be seen with the substance or substances together with an assessment of their severity and a cut off limit of moderate

following treatment (excluding in feed or in water treatment where treatment is repeated over a period of days or weeks) animals will be observed at regular intervals (typically hourly) for the first 4 – 6 hours after treatment, then at 24 and 48 hours should any observations approach the predetermined cut offs then a veterinary surgeon or NVS will be consulted and the animal euthanased, treated and/or removed from the study as appropriate. Observation intervals will be decreased if clinical signs indicate this is necessary.

Anaphylaxis is a rare but possible event. To handle this veterinary assistance, treatment and means of euthanasia will be available in the hours after vaccine administration

Dose volumes and blood sample volumes will follow guidance. The main guidance used will be from LASA and is shown in the table below.

Maximum volume of blood samples

Animal Types	Blood volume (ml/kg bodyweight)	Safe single sample volume	Maximum blood sample per 30 day period	Example maximum blood sample volume per 30 days
Sheep	58-64	6 ml/kg	9 ml/kg	Body weight 50kg - 450ml

Cattle	57-62	6 ml/kg	9 ml/kg	Body weight 500kg - 4500ml
Pig	65	6 ml/kg	9 ml/kg	Body weight 50kg - 450ml
Chicken	60	6 ml/kg	9 ml/kg	Body weight 4kg - 36ml

As small a volume as required for the experiment will be withdrawn. Not more than 10 per cent of blood volume will be removed at one time, and less than 15 per cent of blood volume removed in a 30 day period.

Limit Volumes for Substances Dosed (based on LASA guidance)

*: volume will be adjusted depending on actual bodyweight. Licensed veterinary products will be given at the manufacturer's recommended dose rates and volumes (except in protocol 1).Based on aqueous substances i.e. readily absorbable material (NVS advice must be sought if substances are viscous).

Volumes for pigs should be based on those for sheep.

Admin. Route Species	Oral	Subcutaneous	Intramuscular	Intravenous	Intradermal
Sheep (ml/kg bodyweight)	2 (drench) 10 (gavage) 2	2 (50ml per site)	0.1 (5ml per site)	2	0.001
Cattle (ml/kg bodyweight)	2 (drench)	1 (400ml per site)	0.3 (25ml per site)	1	0.002
Birds (average bodyweight 2.5 kg)	-	1 - 2 ml/site*	0.25 ml*	3 - 5 ml*	-

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild and moderate. 75% mild. 25% moderate (see full breakdown in protocols). The maximum expected severity is moderate with any animals that reach this threshold being treated or euthanased as appropriate before they reach the severe threshold.

Animal types	Est. numbers (mild)	Est. numbers (moderate)	Life stages
Cattle	38	12	Adult and Juvenile

Sheep	75	25	Adult and Juvenile
Pigs	75	25	Adult and Juvenile
Birds	750	250	Adult and Juvenile

What will happen to animals at the end of this project?

- Rehomed
- 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?

Veterinary medicines, biologicals, feed additives, components and contaminants, and vaccines must be trialed in the target species for initial safety/tolerance and pharmacokinetic reasons before taking forwards to larger trials or for regulatory submission. Unless otherwise recommended or if there is an established and validated tissue culture model available, the target animal will need to be used in these studies.

Which non-animal alternatives did you consider for use in this project?

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?

We use a combination of tools to estimate the numbers of animals needed in an individual project. This normally includes the NC3Rs Experimental Design Assistant, VICH and EMA guidelines as well as consulting guidelines set by regulatory bodies and any relevant literature to ensure that the minimum number of animals is used.

Peer-reviewed journals and advice from external peers together with in-house experience and historical data over a number of years is used to ensure that animal numbers are adequate.

We are fully committed to reduction, nonetheless the exact numbers of animals required will vary with the particular study needs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have many years of experience of working with veterinary medicines, biologicals, feed additives and vaccines in the target species. This ensures the minimum number of animals are being used for any particular study. Due to our experience we have highly optimised our processes which enables us to maintain high welfare standards whilst delivering our clients requests. We also utilise the NC3Rs Experimental Design Assistant, VICH and EMA guidelines as well as consulting guidelines set by regulatory bodies and any relevant literature.

For example, pharmacokinetic studies are normally negatively or positively controlled, parallel studies or cross over with a wash out period where the animal is used as its own control.

Expert statisticians will be involved in study design as well as consulting guidelines set by regulatory bodies and any relevant literature to ensure that the minimum number of animals is used that are needed for a valid statistical result.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate pilot studies are used to establish an appropriate and consistent model that will ensure meaningful results are obtained.

Studies may be exploratory and use small numbers in a standardised design, enrolling further animals one at a time as it is established that the adverse reactions to the drug are mild or moderate and not severe, therefore slowly building up the data to a full set.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 species of animals used are the intended target species for each veterinary medicine, biological, feedstuff, feed additive or water additive or vaccine. Regulatory bodies require safety and pharmacokinetic data from the target animal species. The species involved are:

cattle, sheep, pigs, domestic poultry and other farmed avian species (ducks, pheasants, geese).

Models are designed to minimise severity as far as possible to achieve meaningful results. The numbers chosen will normally be within the guidelines set by VICH GL43, GL46, GL52 and EMEA/CVMP/133/99.

Why can't you use animals that are less sentient?

We have to use these animals due to the research being directly related and utilised in real world farm animal and bird environments. However we regularly refine and/or validate existing animal models in support of 3Rs initiatives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We minimise suffering by examining each study plan to identify opportunities to reduce handling by, for example, using experienced and knowledgeable staff, handling and habituating animals to procedures where necessary, meeting or exceeding codes of practice and current guidance or literature suggestions for enrichment.

The animals used are the target species for the veterinary medicines and vaccines on trial. Any models will have welfare at the centre of their design. In addition, animals on these studies will have a heightened level of observations immediately after and in the days following administration of any substance. Our standard level of monitoring is a minimum of twice daily. Frequency of observations are increased on an as needed basis based on animal observations and behaviour. For example, observation frequency would increase to hourly or constant monitoring if necessary. Observations would normally be general health observations but these are supplemented with clinical observations by our NVS (or similar), monitoring of temperature and other parameters as necessary.

If a treatment is to be administered over the course of a number of days then we would aim to administer at the same time daily to allow the animal to become accustomed to the dosing regimen. We may also associate dosing and administration with feeding times as positive reinforcement for the animal

If it is believed an animal is approaching and likely to breach the predetermined severity category then we have a well-established process. This includes seeking veterinary advice from our NVS or another veterinary surgeon, discussions with the NACWOs, PELh, PPLh, PILh and Study Investigator. This group of people decide the best next steps in terms of animal welfare. This could be either, immediate alleviating of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, and increased monitoring if the animal is unlikely to breach the severity limit by the conclusion of the study if appropriate.

If an animal breaches the predetermined severity category for the study then immediate action is taken. This often includes rapid discussions with the NVS, PELh, PPLh and PILh alongside the study investigator. We would also possibly contact an ASRU inspector. The course of action could be immediate alleviation of suffering if irreversible clinical signs via euthanasia, treatment with, for example, analgesics and anti-inflammatories in order to relieve suffering, maintaining and closely monitoring the animal if the scientific need is justified and the end of the study is within a short time frame, this would be agreed by an

ASRU inspector. All decisions are taken on a study by study basis and differs with treatments and species.

We will also continually review our procedures and refine as much as possible with guidance from, for example, VICH, EMA, and other relevant organisations and published literature.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

VICH and EMEA guidelines for best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly reference the NC3Rs website, published literature, attend relevant conferences, and employees are members of The Royal Society of Biology and other relevant organisations.

142. Optimising cardio-pulmonary vascular interventions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Cardiac, Pulmonary, Arteries, Interventions, Monitoring

Animal types	Life stages
Pigs	juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop new, and improve existing, interventions in the arteries of the heart and lungs for patients with coronary and pulmonary arterial disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are two major disease types targeted in this application

Pulmonary hypertension (high blood pressure in the lungs) is a common disorder that leads to heart failure and is associated with high morbidity and mortality. Treatments can be both endovascular and medical. Both need to be carefully targeted and closely monitored. Large animal models play an important role in assessing novel treatments and minimally invasive ways of monitoring progress.

Coronary artery disease (furring up of the arteries of the heart) is the most common serious disease of our times, and in the worst affected cases, surgical bypass or angioplasty (balloon and stent) of narrowed vessels is required. When an artery blocks, the resulting heart attack can lead to extensive heart damage and heart failure requiring cardiac support.

Large animal models are required to optimise interventions for these conditions.

What outputs do you think you will see at the end of this project?

The new information generated will include: the feasibility and safety of novel devices to treat coronary artery disease; biological signals that novel therapeutics may prevent graft failure; physiological measurements to support the translation of devices such as pumps to support the circulation; and metrics of reduced pulmonary artery pressure in experimentally induced pulmonary hypertension.
Outputs will include scientific publications, presentations at medical meetings and conferences, higher degrees for researchers, and the development of intellectual property.

Who or what will benefit from these outputs, and how?

In the longer term, patients with pulmonary hypertension, coronary artery disease, heart attacks, heart failure and cardiogenic shock will see an improvement in their treatments.

How will you look to maximise the outputs of this work?

We will collaborate with relevant researchers and experts in the areas under study, publish our findings in peer review journals, disseminate at specialist conferences; and, if successful, seek to commercialise new developments and have them endorsed by regulatory bodies.

Species and numbers of animals expected to be used

- Pigs: 102

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 generally use juvenile pigs, 15-60kg. Their anatomy and physiology are similar to humans. Our devices, techniques, entry points, instruments and skills work well in the pig. Re-design, repurposing or miniaturisation for other animals would delay research. We have a track record in developing and using porcine models over the last 30 years which have proven durable and effective at testing interventions.

Typically, what will be done to an animal used in your project?

A typical experiment will involve general anaesthesia followed by a vascular procedure (an intervention on the blood vessels). This may involve vascular access (insertion of temporary catheters into the circulation), usually via carotid or femoral arteries or large veins, surgery on the vessels themselves, placement of devices, either temporary or

permanent, in or adjacent to the cardio-pulmonary circulation, monitoring, wound closure and recovery. The experiment will usually end with a second, non-recovery, anaesthetic and further study of the effect of the original intervention before killing the animal. The duration may be from one day to one month between the first and the terminal procedure.

The above basic procedure may be augmented by pre-treatment with drugs to create models of clinical conditions, such as monocrotaline, beta-blockers, vasodilators or vasoconstrictors to induce pulmonary hypertension and/or heart failure. We may then give drugs to optimise the interventions, usually given orally in food, or in select cases by subcutaneous injection.

In a few animals, the above will be accompanied by deliberate coronary vessel occlusion and myocardial infarction, implantation of a left ventricular assist device (LVAD) or circulatory support device in or adjacent to the heart (eg in the pericardium [the space around the heart] or great vessels), followed by wound closure, but with a cable tunnelled under the skin to a power source worn in a harness.

What are the expected impacts and/or adverse effects for the animals during your project?

Typically, there will be few adverse effects. About 10% of recovery animals may have wound discomfort at a vascular access site, treated with analgesics. A proportion of those (5%) may have a wound infection, treated with incision, drainage, antibiotics or killing by schedule 1 method. The few animals undergoing myocardial infarction may experience sudden (usually painless, arrhythmic) death in the first few days. Those having induction of pulmonary hypertension may experience shortness of breath, fluid accumulation and lethargy. Those receiving an LVAD may find wearing a backpack uncomfortable. They will be pre-trained to avoid this. A more serious impact may be that the LVAD or the cable may erode or become infected. In such a case the animal will be killed. We plan to install a camera in the pen to ensure there is no prolonged suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Acute studies are non-recovery. The recovery experiments are moderate, and these generally involve a single procedure under general anaesthetic, followed by a period of recovery and killing under a Schedule 1 method. The LVAD experiment will also be moderate because the animal will be trained to wear the harness. We will not progress from 'non-recovery' to 'recovery' until we are convinced it is safe to do so; i.e. until we have shown that an intervention (such as implanted device, or a graft operation), works without complication for at least 3h under anaesthetic. Therefore the exact numbers of animals in each category are (at the time of writing) as yet unknown; but we will only proceed to non-recovery experiments after discussion with the NVS.

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?

All basic science and bench testing will be complete. This work is designed primarily to test feasibility and safety prior to 'first-in-man' studies.

Which non-animal alternatives did you consider for use in this project?

In the case of devices, all appropriate pre-implantation physical and bench testing will be performed prior to animal work. Biocompatible materials will be used which have previously been tested. In some cases, cadaver testing will be employed, but this is generally neither fully satisfactory nor realistic. In the case of monitoring, only a living being is appropriate. In the case of drugs, they will already have been approved for use or in early phase clinical studies.

Why were they not suitable?

Very often they are suitable, as outlined above, and have already been deployed prior to animal testing, so that animal usage is kept to a minimum.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based upon our group's extensive experience of working with the pig as a model in these fields, under HO Project Licences, over the last 30 years. It is a realistic maximum, but in practice, we usually use fewer animals than this as scientific plans change and grants are (and are not) awarded.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The main method is to ensure that every possible design, engineering, benchtop and computational option has been thoroughly explored before animal testing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In novel device design, we start with animal cadaveric deployment, after another animal experiment is over. We then progress to a procedure under a single, non-recovery anaesthetic. The next stage is implantation with recovery. By employing intravascular implantable monitors, we can maximise data output from few animals over a prolonged period, without requiring more animals to be killed at intermediate timepoints. In this

licence, we will capture extensive haemodynamic and imaging data to maximise the learning from each animal. For novel device therapy, feasibility, or the next design challenge, may only require one animal in a staged experimental design before the next improvement. If a device fails (such as the left ventricular assist device, LVAD, which is technologically complex), and data collection is incomplete, provided the animal is well, and only under the original anaesthetic, we will replace the faulty device. This will avoid the use of another animal. If the device fails once the animal has recovered, this would be a humane endpoint.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Coronary artery intervention

This model is intended to study new devices and technologies and to test their feasibility and safety in patients with coronary heart disease. Under general anaesthesia, we gain access to the circulation and advance the device via catheter to its target vessel, deploying it and studying the response with suitable monitoring. We then withdraw the catheter, repair the wound and allow the animal to recover. In selected cases, two procedures may be required. After waking from the anaesthetic, the animal usually resumes normal activities immediately and is undistressed.

Coronary artery surgery

In the pig we can perform an operation similar to heart artery (‘bypass’) surgery, which used inpatients with more extensive (multi-vessel) coronary heart disease. Instead of instrumenting animal's heart, we can use the blood vessels in the neck. As above, a second procedure may be required to harvest the blood vessels and study the effect of drug interventions. This is minimalist procedure which avoids the invasive trauma of heart surgery whilst yielding the data we need with minimal distress.

Pulmonary hypertension

In a non-recovery study acute pulmonary hypertension may be induced by intravenous administration of a pulmonary specific vasoconstrictor. Over a 5-10 minute timeframe this increases vascular tone in the pulmonary arterial bed increasing vascular resistance and pulmonary pressure leading to right heart strain. Chronic pulmonary hypertension may be induced by sub-cutaneous or intra-peritoneal ammonisation of monocrotaline. This causes a gradual remodelling of the pulmonary arterial bed that takes 7-30 days to increase vascular resistance and pulmonary pressure leading to right heart strain and heart failure at later timepoints. After waking from the anaesthetic, the animal usually resumes normal activities immediately and is undistressed. As heart failure progresses the animal experiences lethargy and shortness of breath. The use of remote monitoring devices

permits close observation of animal activity and physiology thereby ensuring suffering is minimised while extensive, high value data are collected.

Heart failure

A variant of the ‘coronary intervention’ procedure may be used to induce a heart attack by blocking an artery in the heart for 30-60 minutes. The animal has cardiac monitoring, because heart rhythm disturbances can occur which can be immediately reversed with an electric shock under the anaesthetic. Interventions can then be targeted, for example by inserting a novel pump to support the heart. The function of the heart, and any improvement in it, can be monitored. Alternatively heart failure may be induced by intravenous administration of approved drugs that alters cardiac function (beta blockers, vasodilators, vasoconstrictors). Initial testing of the pump can be performed in the animal without a heart attack, but improvement of cardiac output in heart failure will require the heart failure (heart attack) model. This is a challenging set of experiments and will be performed one by one, with full discussion with the NVS and by close collaboration with the Roslin Institute who have suitable facilities to monitor the animals.

Monitoring

All the above procedures may have remote monitoring. A small device may be left in the circulation which will allow heart rate and vessel and/or interstitial pressures to be monitored and transmitted to a receiver. This will provide lots of data from few animals and avoid unnecessary repeated procedures. It will also allow early identification of deterioration and permit intervention or a humane endpoint.

Why can’t you use animals that are less sentient?

These interventions and devices are intended for clinical use, so the main issue is feasible deployment and correct sizing in an appropriate large animal model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our procedures are moderate and the main issue is discomfort from the vascular access site in the recovery period. We try and minimise this with surgical suturing techniques and infiltration of slow- release local anaesthetic. Distress is usually minimal, and close observation at least twice a day in the first three days reveals 5-10% animals that require extra analgesic. Experiments involving a heart attack may lead to sudden (arrhythmic, painless) death in the first 48 hours. We will install a CC camera system to monitor that situation. In the few animals that have a large device implanted, especially those requiring a transcutaneous power supply, such as a left ventricular assist device (LVAD), initial implantations will be under non-recovery anaesthetic; and subsequent recovery animals will be in an establishment with an intensive care facility. Experiments with implantable monitors will provide the data required for us to detect early signs of deterioration before suffering takes place.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk/wp- content/uploads/2018/05/Aseptic-Surgery.pdf)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will meet with our NC3Rs Regional Programme Manager to discuss the latest developments in the 3Rs. Attend local and national 3Rs workshops, seminars where applicable. We will consult the NC3Rs website to keep abreast of any new developments (https://www.nc3rs.org.uk/). We have provided detailed information on advances we have made in the 3Rs area to other groups in the UK who are looking to implement these changes locally.

143. Investigating the mechanisms of hypoxic ventilatory control

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

carotid body, HIF, ventilation, hypoxia

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	adult, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this work is to understand the mechanisms of enhanced ventilatory responses to low oxygen (hypoxia), a process that is mediated by the carotid body.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Oxygen is essential for life and lack of oxygen or hypoxia is associated with many pathologies including stroke, heart attack, peripheral vascular disease, pulmonary hypertension and cancer.

In response to low oxygen or hypoxia, the body mounts various responses to try to adapt to the hypoxic insult such as increased ventilation and red blood production. Hypoxia-inducible factor (HIF) is a master transcription factor that mediates gene expression changes necessary to mount many/most of these adaptive responses to sustained

hypoxia. Previous work has shown that a specific isoform of HIF: HIF-2 is required for the respiratory reflex to low oxygen (hypoxic ventilatory control), both in mediating the increase in ventilation in response to sustained hypoxia (ventilatory acclimatisation) but also in mediating the rapid responses to hypoxia that occur within seconds of hypoxic exposure (acute oxygen sensing).

This project will seek to understand the mechanisms of action of the respiratory reflex to low oxygen (hypoxic ventilatory control) that is mediated by the carotid body, in response to both sustained (ventilatory acclimatisation) and rapid (acute oxygen sensing) exposure to hypoxia. This is important both scientifically, in understanding the mechanisms involved in this signalling pathway (in particular in elucidating the mechanisms of acute oxygen sensing, which remain unknown) as well as biologically, since HIF-2alpha antagonists are clinically licensed for use in treating kidney (and other) cancers and our work shows that these drugs likely have adverse effects on ventilatory control, contraindicating their use in patients (eg with impaired lung function) that rely on hypoxic ventilatory control for adequate oxygenation of tissues.

What outputs do you think you will see at the end of this project?

We aim to obtain the following outputs from this work:

an understanding of the mechanisms of hypoxic ventilatory control (in particular acute oxygen sensing, which are hitherto unknown)

publications in peer-reviewed journals

to better inform the use of the clinically licensed HIF-2alpha antagonist (Belzutifan), which has recently been licensed by the FDA to treat kidney and other cancers yet likely has adverse effects on hypoxic ventilatory control may lead to a novel use of the clinically licensed HIF-2alpha antagonist (Belzutifan) in the treatment of carotid body and related tumours (pheochromocytomas and paragangliomas)

may lead to the development of new drugs targeting respiratory control, relevant to patients eg with impaired lung function such as chronic obstructive pulmonary disease, obstructive sleep apnoea/metabolic disease.

Who or what will benefit from these outputs, and how?

The findings of this proposal will benefit:

the scientific community, in understanding the mechanisms of hypoxic ventilatory control, in particular the mechanisms of acute oxygen sensing

patients being treated with the HIF-2alpha antagonist Belzutifan for kidney and other cancers, in better understanding the adverse effects on respiratory control

patients with cancers of the carotid body, other paraganglia and related adrenal medulla (pheochromocytomas and paragangliomas, PPGLs), in understanding whether the use of HIF-2alpha antagonist Belzutifan extends to these types of cancer
patients with respiratory diseases eg chronic obstructive pulmonary disease (COPD) who rely on hypoxic ventilatory control for adequate oxygenation of their tissues

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised by:

dissemination of results by collaboration with other research groups (both internal and external to the establishment)

oral/poster presentation at conferences and publication in peer-reviewed journals

publications of results in peer-reviewed journals

Species and numbers of animals expected to be used

- Mice: 10,000
- Rats: 800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures. Explain why you are using these types of animals and your choice of life stages.

The proposed work requires an animal model which has a carotid body, lungs, brain and nervous system in order to carry out hypoxia induced ventilation. The carotid body is only present in (adult) vertebrates and the mouse is the lowest vertebrate with all the necessary multi-organ systems required to mount a hypoxic ventilatory response. Additionally, genetically modified mouse models already exist for much of the required work. As such, this work will primarily involve the use of adult mice.

Since the carotid body is small (~1000 cells in mice) and difficult to dissociate into cells, we will additionally use carotid bodies from adult/juvenile rats.

Typically, what will be done to an animal used in your project?

Animals will undergo administration of a gene-inducing or deleting agent over a period of typically five days and by oral gavage. They are then acclimatised to hypoxia, or air containing ~10% oxygen (normal air has ~21% oxygen), which is equivalent to oxygen levels at ~5500m altitude. They are then housed for up to 7 days in these conditions, during which their ventilation in response to normal air and acute (5 mins) hypoxia (10% oxygen) will be measured whilst animals remain conscious. At the end of this period, animals will be killed and tissues harvested for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals typically dehydrate and lose ~5-10% of their body weight within the first 48 h of hypoxic exposure; animals usually recover body weight shortly after or 48 h post commencing hypoxic exposure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse: mild (90 %); moderate (10 %) Rats: mild (90 %); moderate (10 %)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this work is to understand how the body adapts to low oxygen (or hypoxia) by increasing ventilation; this process is mediated by the carotid body. Since ventilation is a systemic/multi-organ response involving the lungs, heart, blood, brain, carotid body, nervous tissue and other organs, it is not possible to study this process without the use of animal models. Additionally, the carotid body is present in vertebrates but not lower organisms. Since mice display hypoxic ventilatory responses mediated by the carotid body and many genetically modified mouse lines are already available for this work, they are the ideal model system for this study.

Which non-animal alternatives did you consider for use in this project?

In vitro cell lines that are kept in tissue culture.

Lower organisms including the fruit fly D. melanogaster, the nematode worm C. elegans. These are widely used in research since they are easy to modify genetically.

Computer modelling.

Why were they not suitable?

Cell lines, that is isolated cells that are cultured in the laboratory, are not useful for studying whole body, multi-organ systemic responses such as ventilation, in particular ventilation that is induced by hypoxia and is mediated by the carotid body. However, cell lines will be useful to complement the animal work and to dissect the exact mechanisms behind carotid body Type I cell responses to hypoxia.

Neither D. melanogaster nor C. elegans have carotid bodies or display hypoxic ventilatory responses and are therefore not useful animal models for this proposed work.

Computer modelling cannot be used to simulate breathing responses to hypoxia.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design

studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 studies into hypoxic ventilatory control. These studies demonstrate that these numbers are sufficient for the studies to be valid.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments were designed using NC3R's Experimental Design Assistant to ensure use of animal numbers are minimised and consistent with the scientific objectives, methods to reduce subjective bias and appropriate statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Number of animals used in this project will be minimised by various strategies including:

use of efficient breeding programmes to ensure that the genetic status of offspring generated are suitable for our experiments

performing pilot studies using small numbers of mice to allow for adjustments to the protocol without unnecessary wastage of mice

sharing of tissue from one mouse between several users (internal and external to the research group) wherever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice (and to a lesser extent rats) will be used for the work proposed in this licence. Hypoxia will be simulated using a normobaric (ie normal pressure or barometric pressure equivalent to pressure at sea level) altitude chamber and ventilation measured using plethysmography.

Why can’t you use animals that are less sentient?

Hypoxic ventilatory responses require the animal to have a cardiopulmonary system, carotid body (only present in vertebrates) and nervous system. Therefore, mice (and rats)

are among the lowest vertebrates to mount hypoxic ventilatory responses to hypoxia which can be used for study.

The carotid body forms post-natally and therefore these studies require the use of adult mice and rats. Additionally, we would like to look at carotid body function/hypoxic ventilatory control during development, thus requiring the use of juvenile and neonatal mice and rats.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Potential refinements to reduce welfare costs to the animals include:

enhanced monitoring, for example, when animals are under hypoxia they will be monitored (including measuring body weights) once daily and animals provided with mash if losing >10% body weight.

training animals where appropriate to reduce stress caused by the experiment

use of conditionally inactivated transgenic animal models to avoid adverse effects of ubiquitous gene deletion in development

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be performed in accordance with published literature on refining experiments, for example, the NC3R's website, Arrive Guidelines, RSPCA, the 'Handbook of Laboratory Animal Management and Welfare' by Wolfensohn and Lloyd.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through regular attendance to internal welfare meetings and collaboration with colleagues.

144. Development of novel cancer therapeutic 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

cancer, treatment, resistance, toxicity, efficacy

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?

More than 1 in 3 of us will develop cancer during our lifetime. The aim of this project is to find novel treatments and to determine their properties prior to their evaluation in the clinic.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Current therapies are associated with non-selective side effects and tumours can develop resistance to the drug resulting in the death of the patient. Consequentially, there is an urgent need to develop targeted therapies to each type of cancer.

What outputs do you think you will see at the end of this project?

Publications in peer-reviewed journals.

Compounds or drugs that have efficacy in animal models that could progress to clinical development.

Who or what will benefit from these outputs, and how?
Many of us are likely to develop cancer during our lifetimes. The ultimate output benefit will be to the cancer patient. New therapeutic agents have the potential to improve their prognosis. Thus addressing high unmet clinical need. Current agents are associated with both a a poor efficacy signal and the emergence of side effects. In addition, tumours can develop resistance to pharmacotherapy. In the shorter term, scientific knowledge and understanding of pharmacology of cancer chemotherapeutic agents will be increased and agents will be developed that can have greater efficacy and/or an improved side effect profile.

How will you look to maximise the outputs of this work?

I publish regularly in peer-reviewed journals and present at national and international meetings. I also collaborate with academic groups and both biotech pharmaceutical companies. Thus new knowledge will be disseminated.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The adult mouse is well validated as a model for the development of cancer therapeutic agents. Immunocompromised and immunocompetent mice will be used for the growth of human and mouse tumours respectively.

Typically, what will be done to an animal used in your project?

The majority of mice will bear a tumour, typically on the flank, but also directly into the tissue of origin. Following growth of tumours (2-4 weeks), mice will be treated with chemotherapeutic agents for up to 4 weeks, with blood samples being taken where required.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals are expected to tolerate both the tumour and treatment well. In the minority of cases, tumour burden will result in some weight loss, up to a maximum limit of 20%. Intervention would take place if either the tumour burden or treatment affected animals welfare, typically within 8 hrs of observation of 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 animal type)?

We anticipate that the majority of animals will experience moderate severity as a consequence of bearing tumours.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst in vitro systems provide us with important data on the pharmacokinetic (PK) and pharmacodynamic (PD) properties of novel agents, it is imperative for the pharmacological properties to be determined in the intact biological system, e.g. the mouse prior to progression towards the clinic. In vitro systems model parts of the in vivo biological processing of agents, e.g. isolated liver cells to model conversion of the agent to an inactive product or cancer cells to model the activity of the therapeutic agent. However, it has become clear that these data are not sufficient alone to predict effect of these agents in humans. Furthermore, in order to understand the toxicity of these therapeutic agents, an intact biological system is needed to identify and explore unexpected effects on target and non target organs. We would replace animals wherever possible, e.g. numerous biochemical and in vitro cellular assays will be used to select the optimal agents, for progression into in vivo studies.

If we did not use animals for these studies there would be two main consequences: firstly agents with sub-optimal properties would be administered to sick cancer patients, which would be ethically unacceptable and secondly, potentially toxic agents would be administered to patients, who are already unwell as a result of their disease state.

Which non-animal alternatives did you consider for use in this project?

Organoids and other 3D cell culture systems.

Why were they not suitable?

Currently they are poorly predictive of clinical efficacy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on typical study design and number of projects predicted over the next 5 years and underpinned by knowledge gained through 20 years of experience of completing similar studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Extensive previous experience of designing studies of this type.

The models are reproducible, so we are able to use the minimum number of animals.

The minimum number of animals used is also underpinned by an understanding of the variation between animals, with statistician advice sought when needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies are frequently used when developing models and sharing of model information with collaborators 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In terms of welfare the least invasive model will be used in the first instance. Similarly for administration of therapeutic agents, the least invasive route, e.g. oral, will used where possible. Mice will be monitored closely daily for any adverse effects of treatments.

Why can’t you use animals that are less sentient?

The mouse is used for evaluation of new cancer chemotherapeutic agents as it is well validated both in the scientific literature and by the pharmaceutical industry. Tumours derived from human cell lines can be readily grown in immunocompromised mice and this provides a means of demonstrating proof of concept of the agents of interest. Mice are well established for such procedures, relative to a simpler organism such as the fish, due to the intact immune system (for immunocompetent mice), circulatory system and presence of mammalian organs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continually review our peri- and post- operative care procedures and each of the PPLs learn from each other with respect to procedures and refinements. Our NACWO/NVS/NIO attends regular national meetings and provides us with feedback on learnings.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We focus on best practice guidance published by Workman et al., Br J. Cancer, 2010. In addition, regular literature reviews will be completed to ensure that our work aligns with current best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By regular discussion with the NIO, NACWO and NVS in addition to receiving regular updates from NC3Rs. 3Rs are also an item agenda during our quarterly AWERB meeting.

145. Generation, maintenance and archiving of genetically altered mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Disease model, Genetically modified mouse, Embryonic stem cells, Embryo transfer, Cryopreservation

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To generate, breed, distribute and archive genetically altered mice and to supply them for biomedical research.

To develop and implement new technology for the genetic engineering of the mouse.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 modified mice help advance our understanding of normal and pathological biological processes, revealing new targets for drug discovery. They serve as models for human disease, aiding the treatment and prevention of human disease through discovery and development of novel therapies and diagnostic tools.
What outputs do you think you will see at the end of this project?

New insights into the function of genes or the significance of mutations in health and disease

New mouse models of human disease

Refined and more accurate technology

An archive of cryopreserved gametes and embryos

Who or what will benefit from these outputs, and how?

Genetically altered mouse models generated could provide short-term impacts in our understanding of disease biology, the assignment of novel targets for future drug discovery and a basic scientific understanding of normal and pathogenic biology. Long-term impacts would concern the use of the genetically altered mouse models for preclinical drug discovery and novel diagnostic tool development.

With respect to technology development, short-term/medium term benefits would be more predictable and accurate models, reducing the variation in outcome and thus reducing the number of mice that need to be bred and analysed in an experiment. The archive of cryopreserved gametes and embryos has the short-term benefit of avoiding redundant breeding of genetic models when there is no active experimental need for their continued use. Medium term benefits thus amount to a reduction in animal numbers and considerable savings in research costs.

Biomedical research groups focused on understanding biology and treating human disease will benefit from the outputs, offered as a service. The service work carried out under this license will supply mice that will be used on other project licenses that have had their harm-benefit positively assessed by the Home Office. The work on this license will therefore also contribute, indirectly, to the scientific benefits accrued by these other licenses that receive mice from this license.

How will you look to maximise the outputs of this work?

We plan to publish our technical development, both positive and negative, in peer-reviewed journals and at scientific and technical meetings. Genetically altered mouse models that are generated on this license are transferred to end user licenses, and published once the models are fully characterised. We track all models generated to ensure publications result.

Species and numbers of animals expected to be used

- Mice: 28700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages?

Mice are the animal of choice for this project as more is known about their genetic makeup than any other animal and despite appearances, they are genetically very similar to

humans. The mouse can serve as an accurate model of human disease - more often than not, mutations that cause disease in humans, result in similar pathologies when the equivalent mutation is introduced into the mouse.

Embryonic, neonatal and adult life stages will be addressed in this project, as gene mutation can have consequences on all developmental stages of the animal.

Typically, what will be done to an animal used in your project?

Female mice will be hormonally stimulated by two intraperitoneal injections, and sometimes bred with male mice. Mice will be killed and oocytes or preimplantation embryos isolated. Embryos and oocytes will either be cultured and/or genetically manipulated for example by microinjection or electroporation, or oocytes will be used for in vitro fertilization. Female mice will be anaesthetized and embryos surgically implanted. Male mice will be anaesthetized and vasectomized. Both male and female genetically altered mice will be bred to obtain appropriate genotypes and maintained to up to 15 months. Substances may be administered to mice by injection, gavage, or in the drinking water to induce genetic manipulation at a specific time point or developmental stage.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice undergoing hormonal stimulation or induction of a genetic alteration will experience no more than transient discomfort and no lasting harm. Mice undergoing surgical procedures for embryo transfer and vasectomy will experience short-lived post-operative pain and discomfort, which will be ameliorated by the use of appropriate analgesia. The majority of the mice with genetic alteration will be indistinguishable from normal mice, but certain gene mutations could lead to long-lasting mild suffering e.g. behavioural changes, altered immune function, sensory disturbances or weight gain/loss.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% subthreshold

35% mild

15% moderate

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand the complexity of biological processes and to model human disease, it is not possible to rely on cell-culture or computer simulations alone. The effects of genetic alteration on a living organism can be essential to understand the complex interactions that occur. For example when trying to understand neurodegeneration, it can be important to study the behaviour of mice, which can only be done using live animals.

Which non-animal alternatives did you consider for use in this project?

We actively pursue the use of stem cells to model human disease in the dish, and apply many technologies for genetic alteration in this system to explore gene function and mutation. In addition, human patient data can provide information about the significance of mutations or the likely role of particular genes.

Why were they not suitable?

Stem cells can be differentiated to mature types of cells, e.g. neurons, heart muscle cells, but the cells produced are immature and more similar to embryonic stage cells. Experimental results obtained with stem cell-derived cells may not be accurate reflections of the situation in adults. Furthermore, certain biological processes cannot be easily modelled in vitro, for example aspects of the immune system or any brain-behaviour studies.

Human patient data is informative but it is an observational science and discoveries cannot be experimentally validated. A model system for experimental manipulation is thus 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?

Providing this service to the institution over the past 15 years has allowed us to quantify the service need and thus to estimate the number of animals we will require for the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The BVAAWF/FRAME/RSPCA/UFAW Joint Working Group framework guidelines (Altern Lab Anim. 2004 Jun;32 Suppl 1A:373-5) has been used to ensure that the number of animals used for the generation of genetically altered mice is appropriate.

For experimental proof-of-concept work, the ARRIVE2.0 guidelines will be followed and comprehensively reported in resulting publications.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The number of mice have been reduced by maximizing the efficiency of the various experimental steps. The age, strain and commercial source of mice undergoing hormonal stimulation for the generation of good quality embryos have been optimized to ensure a maximal yield per female mouse. Specifically, female mice at under 28 days of age will be used, as this ensures at least double the yield of embryos when compared with using older mice and in vitro fertilization efficiencies are higher when using oocytes from mice of this age (Kolbe et al., 2015 Lab Animal volume 44, pages 346–349).

Similarly, for the procedures that achieve the genetic manipulation of the embryos, the quality and validation of the stem cells and injection material used has been optimized to reduce the number of embryos that need to be manipulated, which then reduces the number of embryo transfer procedures that need to be performed. Breeding procedures have been optimized to ensure no redundant breeding and the efficient use of the mice generated.

Embryo and mouse production and breeding will be tracked using an online database that helps monitor the efficiency of the experimental work.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will predominantly use inbred or hybrid strains of laboratory mice that are of defined genetic background and are not associated with any harmful effects. When mutations need to be generated on a pre-existing disease model, for example a non-obese diabetic model , we will select only established models at the appropriate age before the onset of any pathology, thus avoiding any pain, suffering, distress or lasting harm.

The surgical methods we will perform - embryo transfer and, on occasion, vasectomy - will be performed using the most refined and appropriate aseptic technique. Where appropriate non-surgical alternatives to these procedures will be employed, for example non-surgical embryo transfer when transferring late-stage embryos, or the use of naturally sterile mice.

Why can’t you use animals that are less sentient?

A mammalian model is required for an accurate modelling of many aspects of human health and disease, e.g. immune function, psychiatric/behavioural research, mammary biology etc. Immature life stages cannot always been used as adult stages are necessary to explore biology associated with diseases that manifest later in life.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgical procedures will be performed with peri- and post-analgesia, and post-operative care and regular monitoring will be ensured. Surgical vasectomy will be refined by the use of naturally sterile mice. Surgical embryo transfer will be refined by the use of non-surgical techniques, where possible, for later stage preimplantation embryos.

For technology development, gene manipulations that are associated with simple non-invasive read- out e.g. coat colour will be used for proof-of-concept models.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance and reports from the NC3Rs, norecopa and Laboratory Animal Science Association, including their “Guidelines on Aseptic Surgery”. In addition, we will follow the appropriate newsgroups and publications of the International Society for Transgenic Technologies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are very active in contributing to and attending 3Rs events, both locally, but also nationally e.g. Laboratory Animal Science Association and Internationally e.g. GV-SOLAS (Germany), International Society for Transgenic Technologies Annual Meetings.

146. Role of autophagy during tumourigenesis and tissue homeostasis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Tissue health, Cancer, Treatment, Cellular pathways

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall purpose of this project is to investigate how tissue health is maintained by cellular degradation pathways and how they implicate tumour initiation, maintenance and treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 normal cells in our body function and what goes wrong in diseased cells (e.g. cancer cells) are important to develop better treatment options for various illnesses. This project aims to study one cellular mechanism, known as autophagy, that maintains normal activities of cells but can also be disrupted during disease.

What outputs do you think you will see at the end of this project?

This project aims to better understand how cells maintain their normal function and prevent the onset of disease. The project will also investigate how diseased cells, such as in cancer, can grow uncontrollably and resist therapy. Success of this project will lead to publications and the potential identification of better treatment options.

Who or what will benefit from these outputs, and how?

The proposed studies have a wide range of academic beneficiaries both within the same field and from other disciplines as well as local and external institutions. These are described as follows:

Members of the academic community, particularly those involved in autophagy (a cellular mechanism involved in recycling of material) and cancer research, are a target audience that will directly benefit from outcomes of the proposed work. The project will primarily increase scientific knowledge in these fields. The findings from this study will be disseminated through publications, meetings and seminars, which are expected to take place within the 5 year time period of this study.

The proposed study will also generate valuable and broadly applicable research tools that will be of interest to the wider research/healthcare community. The research tools include a mouse strain that allows temporal or tissue-specific regulation of genes and cancer-initiating cells which have been extensively characterised (genetically and molecularly). On the longer time scale towards the end of this project, we expect that following deposition of generated tools, models and reagents in established and easily accessible repositories there will be lasting impact and availability of these valuable resources for future projects facilitating reproducibility and extension of the work in this field.

Detailed molecular studies of the tumours derived from the animal studies and their relationship to specific genetic mutations involved in human disease have direct clinical impact and can improve treatment strategies and the understanding of survival pathways.

Clinicians interested in cancers (particularly brain cancers) and novel approaches to cancer therapy and personalised treatment will also benefit. This is largely due to the potential of repurposing drugs that can regulate autophagy or key pathways that are regulated by autophagy (identified through our wet lab studies).

In the long term we will partner with pharmaceutical companies to develop and test the benefits of identified autophagy inhibitors in the clinic (potentially beyond the time period of this application).

How will you look to maximise the outputs of this work?

The outputs of this work will be optimised through various ways:

Collaborations with labs that have established expertise in the proposed work (e.g. model generation, methods or data analyses) are currently ongoing as necessary.

Dissemination of research output prior to publication (for e.g. via meetings) is crucial to ensure progress of work and optimal use of methodologies and reagents. Publication of established work will be important to facilitate the research of others and avoid redundancy.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice in our research as they have been extensively utilised and can closely recapitulate human disease. Numerous well characterised mouse models exist to study cancer biology and various forms of disease. Mice will be used at neonatal, juvenile and adult life stages. Neonates will be mainly used in experiments involving intracranial tumour initiation where gene expression is optimal allowing efficient uptake of exogenous agents. Juvenile and adult mice will be used for most experiments involving transgene induction, disease development and drug treatment.

Typically, what will be done to an animal used in your project?

Animals used in this project will undergo injections of substances (through various routes) and will be culled using established humane methods. Typically, animals will be injected with cells (for example cancer-initiating cells) intracranially and monitored for signs of tumour development. These include neurological signs, lethargy, poor grooming, reduced movement and hunched posture.

All experimental animals will be closely monitored for any adverse effects and will be culled immediately upon the onset of harmful phenotypes. Experimental durations will be short terms (around 1 week or less) and up to several months (up to 11 months of age). Animals will be analysed by imaging (under anaesthesia) and their tissues harvested upon culling.

What are the expected impacts and/or adverse effects for the animals during your project?

All methods used in this project will follow established techniques to minimise the impact on the animal’s welfare. Most injections will be conducted under anesthesia and analgesia will be used to reduce any post-surgical pain.

The main adverse effect of this project is tumour formation which will be induced in a way to closely resemble what is observed in the clinic. Mice will be monitored closely for signs indicative of disease formation and will be culled immediately to minimise their suffering. Veterinary surgeons and experienced animal care staff are always available for advice and help since the welfare of the animals is of major concern to us. Despite optimised injection protocols performed by other groups to obtain efficient tumour growth and minimise adverse effects on the animals, there is a potential for some mice to experience sudden death due to cerebral tumour development without prodromal clinical signs. We do not expect this to exceed 5% of the animals injected with tumour-initiating cells (our current injections in neonates resulted in an incidence of ~2% sudden death during >1 month after initial injection). All experimental animals will be culled at the end of the experimental duration.

The majority of substances administered will already be in use in rodents studies and have information on doses and adverse effects available. We will make use of the information. In cases were adverse effects are not known, animals will be closely monitored following treatments in line with advice from local vets.

Imaging techniques are not expected to cause adverse effects other than those associated with anaesthesia. Animals will be closely monitored while undergoing imaging and body temperature will be maintained.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severities will range from moderate to mild for all procedures involving mice in this project, based on previous research 20% animals experience moderate severity and 80% experience mild. Unexpected deaths as a result of surgical intervention will be monitored and appropriately reported.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Various aspect of disease biology, such as tumour microenvironment and metastasis can strongly influence tumour growth and response to treatment in patients. While some of these aspects can be mimicked using in vitro studies using cell culture systems (e.g. anchorage independent growth in soft agar, oxygen and nutrient withdrawal, and transwell migration assays), accurate modelling of tumour growth cannot be recapitulated using non-animal systems. This is especially important when studying cellular processes which are frequently activated under many of the conditions observed in vivo.

Which non-animal alternatives did you consider for use in this project?

Human- and mouse-derived ex vivo tissue investigations and production of long-term cell culture lines, which may ultimately replace some of our in vivo model systems. We have recently considered using patient-derived stem cells to investigate tumour growth pathways.

Why were they not suitable?
The ex vivo models exclude all potential extrinsic factors that influence disease development and are likely only models in complex organisms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate have been derived from previous studies in the lab and based on publications utilising similar proposed models. To stay updated on optimized experimental design, we will regularly seek guidance from biostatistical experts and estimate provided by local animal facility.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will utilise mouse models that ensures highest tumour incidence. Pilot experiments will help optimise the number of mice used and time points. The experimental group size will be determined in order to obtain sufficient numbers of tumours of a measurable size suitable for statistical analysis. We will use genetic systems to introduce genetic alterations (e.g. inhibition and/or activation of cancer- associated pathways) in postnatal animals that avoid the need for breeding thereby reducing mice numbers (breeding of different genetic backgrounds results in variable offspring genotypes some of which are not suitable for experimental use).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies whenever possible to assess the potential for publishable results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen mice to use as they are the animals that are most similar to humans that are readily amenable to genetic modification – this means that, for us, they are the best model to answer our questions about cancer development and therapy. Of specific research importance are cancers of unmet clinical need including aggressive brain tumours. Researchers have developed the most refined models to study brain tumours and patient-derived gliomas are one of the few human cancers for which we can isolate, culture, and genetically manipulate primary cells as well as their genetically "normal" counterparts.

The protocols are designed to generate as much information as possible from as few mice as possible with the least harm possible. They are optimised to obtain efficient tumour formation with minimal surgical intervention and animal discomfort. Anaesthetics are used for any procedures that would be expected to cause temporary discomfort. We are experienced in most of the protocols listed and know them to create minimal suffering. Where we intend to perform a protocol that we are less familiar with, we do so in consultation with the veterinary surgeons so that suffering can be minimised.

Models that use exogenous vectors to introduce genetic alterations will follow minimally-invasive protocols that ensure least discomfort in animals.

Why can’t you use animals that are less sentient?

Tumour development and changes in tissue homeostasis occur over time and require periods ranging from few weeks to months (depending on the model). Therefore, disease progression will require monitoring mature animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Any of studies involving surgical or invasive intervention will adopt appropriate pain management and post-operative care.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rs website (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about the advances in the 3Rs by attending informational events provided locally and provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). Annual 3R-related symposia are also locally organised to help researchers share best practice and contributions to the 3Rs across various disciplines.

147. Experimental therapies in aggressive variants of prostate cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

prostate cancer, small molecule inhibitor, targeted therapies, Patient-derived xenografts, Genetically engineered mouse models

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to use animal models to test the safety and efficacy of experimental therapies for an incurable and common form of 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.

Why is it important to undertake this work?

Early prostate cancer is curable through a combination of local therapies and hormonal therapies. However, several years after treatment, some prostate cancers become progressively more resistant to any treatment and invariably fatal. These cancers are

called "aggressive-variant prostate cancer" (AVPC). There is currently no treatment for this type of prostate cancer. Here we propose to use clinically relevant animal models to test new treatments for AVPC. This will be an essential step for starting clinical trials and eventually improving the life expectancy and the quality of life of these patients.

What outputs do you think you will see at the end of this project?

Our study will allow us to test different therapies in animal models of AVP, and to assess their effects on tumour growth and on key molecular markers. Hence, these studies will provide biological insights into the molecular mechanisms of prostate cancer progression.

We expect to publish at least two peer-reviewed experimental articles deriving from these studies (one per tested compound).

We expect to obtain key information on the safety and efficacy of experimental therapies in clinically relevant animal models of AVP. This information will inform future clinical trials.

Who or what will benefit from these outputs, and how?

In the short term (2-5 years), these studies will provide insights into the molecular pathogenesis of prostate cancer, which will be published in peer-reviewed journals. Hence, beneficiaries of this projects will be other scientists and clinicians working in this field.

In the long term (5-10 years), we hope that the primary beneficiaries of these studies will be patients with incurable prostate cancer. If these studies allow us to identify promising drug candidates, we will work with our clinical collaborators to design clinical trials. Our final goal is to improve the life expectancy and the quality of life of prostate cancer patients.

How will you look to maximise the outputs of this work?

All our data and publications are freely accessible via the University portal. We will deposit all the publications in this portal, and link genomic data to appropriate servers. This will maximise the reach of our discoveries in the scientific community.

We plan to present and discuss the data at different meetings, e.g. the European Society of Medical Oncology congress, the Prostate Cancer Foundation Retreat (the Applicant has been invited to both types of meetings in the past).

We plan to use these data to strengthen our collaborative network, which include leading clinical centres in the UK and abroad. Results will be discussed with these collaborators, and where appropriate we will take steps towards clinical testing and applications.

Species and numbers of animals expected to be used

- Mice: 980

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cancer models based on cell lines are useful for initial screening of drug activity, toxicity and mechanism of action. However, these in vitro models do not recapitulate the clinical complexity of the disease (e.g. they do not account for tumour-microenvironment interactions). Hence, restricting our studies to these in vitro models might not fully elucidate the clinical efficacy of the experimental drugs. In our study, we will be using human and murine prostate cancer cells, implanted into mice. These models provide a platform for finding novel therapies to treat human patients afflicted with prostate cancer. The two strains of mice will be in their adult life stage to accommodate the size of the tumour.

Typically, what will be done to an animal used in your project?

Animals will be injected with prostate cancer cells (sub-cutaneous injection, flank) and monitored for health and tumour growth.

Animals will be randomised to receive different treatments, typically via intra-venous injections. Typically, animals will be maintained for up to 12 weeks and then humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Likely adverse effects will be mainly due to the tumour (possible weight loss, ulceration), while the treatment is expected to have mild side effects. Animals will have surgery to implant tumours just under their skin. During this procedure, the animals will be unconscious (under anaesthesia), and the recovery will be aided by giving animals analgesics and keeping them warm. The level of pain the animal will experience after the surgery will be moderate and short-lasting. There also could be a mild, long-lasting pain that may accompany the tumour growth. Animals will be euthanised at the end of the experiment or when adverse events occur, and the pain, discomfort or suffering are not controllable

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We will be using mice and expected to have moderate severities. 60% of mice will have moderate severities, and 40% will have mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Clinically relevant animal models enable experimental studies that can predict drug efficacy and safety in humans and reveal new information on their pathogenesis. Unlike in vitro models, animal models enable the study of the complex interaction between the tumour and its micro environment. Animal models also enable the study of key pharmacological characteristics of the experimental drug, e.g. its absorption, distribution in different tissues and stability in biological fluids.

For this particular study, we will employ murine cancer models. Mouse modelling has made a significant contribution to the study of prostate development and disease. The reasons for this success include:

the mouse and human genomes share more than 90% of protein-coding genes; most cancer relevant genes are highly conserved in mice and human.

mice are relatively easy to genetically modify; this allows for the generation of immunocompromised models that can host patient-derived xenografts (PDXs), and for the generation of cancer tumour allografts in immunocompetent mice (mice cancer cells implanted in syngeneic mice), which recapitulate key characteristics of the tumour micro-environment. mice have a relatively short gestation time and are small, they are reasonably easy and affordable to house and breed to generate large populations.

It has been demonstrated that murine PDXs and allografts are superior to currently available in vitro models: these systems can accurately predict key features of the human cancers, including efficacy and safety of experimental therapies. Here we propose to use the two complementary models to test our experimental therapies as accurately as possible.

Which non-animal alternatives did you consider for use in this project?

We have considered relevant websites (NC3R, FRAME) to identify alternatives to the use of animals in this specific research project. Considered alternatives included spontaneous tumour models in mammals and tri-dimensional patient-derived cancer cultures. Patient-derived cell lines will be used to perform initial screening of anticancer activity and toxicity. Only the most promising compounds will be then tested in vivo.

Why were they not suitable?

Unfortunately, none of the alternatives proposed allows for reproducible and controllable results and/or for accurate reproduction of a human neoplasm's clinicopathological characteristics. In particular, spontaneous cancer models of NEPC are extremely rare and unpredictable (variable tumour size, growth, and metastatic rate); tri-dimensional cancer cell cultures are promising, but they do not allow for appropriate pharmacokinetic and pharmacodynamic analyses, and there is no definitive evidence that they can predict patient's response to therapy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These 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 a typical set of experiments, we would test up to 4 experimental drugs in separate experiments with randomisation and appropriate controls. We have planned for pilot studies for each of these treatments. Each pilot study will employ approximately 9 mice (control and 2 doses of the drug). Hence we will need up to 36 mice for pilot studies.

For the main studies, we have calculated that we will need approximately 20 animals per treatment group. This estimate is based on a sample size estimation conducted by our collaborators with bio- statistical expertise; based on the spontaneous variance of PDX tumour weight in prostate cancer models. Since we will need a total of 8 treatment groups (4 control and 4 experimental therapies) the total number of animals for the main studies is 160 (maximum). We anticipate running up to 5 sets of
experiments during the 5 years. When different therapies will be tested at the same time, we will endeavour to use one control group for multiple treatments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will employ the minimum number of animals while ensuring that scientific objectives are met. To this end, we have conducted sample size estimations, which are based on our previous experience in animal research, and on the extensive experience of our collaborators that have generated the in vivo models.

In addition, all the compounds selected for in vivo study will undergo extensive preliminary in vitro tests. Each drug candidate will be screened in a panel of normal and prostate cancer cell lines, to determine: cytotoxicity, effects on cell cycle and apoptosis and reduction of the target gene expression/activity (depending on the proposed mode of action). Compounds will be selected for in vivo studies if they meet the following criteria, based on in vitro experiments: (I) differential toxicity in cancer vs normal cells (at least twice as toxic in cancer vs normal cells); (II) confirmed inhibition and/or silencing of the validated therapeutic target; (III) activity at clinically achievable concentrations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tumour-bearing animals will be matched by tumour size and randomised to administer treatment vs control drug. We will analyse tissues from previous experiments and from collaborators whenever possible (e.g. to confirm the expression of the target gene before selecting the appropriate in vivo model for our experiments). Data generated by this project will be publicly available. This will reduce the need to repeat experiments in the future.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will employ 2 mice models in which cancer cells are injected under the skin (flank) of a mouse: an immunodeficient mouse model for patient-derived xenograft (human tissue injected in mice) or immune-competent mouse models (murine prostate cancer cells injected in mouse). The flank injection enables tumour monitoring with minimal impact on the animal wellbeing and locomotion. We will follow appropriate guidelines to minimise animal distress by using humane endpoints. Test compounds will undergo extensive testing in vitro to determine efficacy prior to any in vivo studies. Dose rates and route/frequency of administration will be determined from available literature, if available, and from our in vitro studies. For compounds where no in vivo data is available, we will conduct small pilot studies to determine optimum dosing regimes before proceeding to full studies.

Why can’t you use animals that are less sentient?

Whilst most cancer-relevant genes are conserved between mice and humans, this is unfortunately not true for less sentient species. There are several similarities between mouse and human immune system and blood vessels, which are not conserved in other (less sentient) animal models employed in biological research (e.g. C. elegans and Drosophila melanogaster). Moreover, it is not known whether clinically-relevant mammalian prostate tumours can be grown in non-mammalian hosts, for the purposes of this study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will critically evaluate our practices on regular basis and we will constantly seek ways of conducting our research in a manner that will allow us to maximise our scientific output with minimal impact on the animal's welfare. We will apply the refinement loop technique described by other scientists, to improve all aspects of the animal's use. We will identify appropriate parameters to assess the well-being and needs of the animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
Daily mouse appearance, activity, posture and other signs (including ulceration) will be scored, as recommended (CRUK guidelines: https://www.ncbi.nlm.nih.gov/pubmed/20502460). Administration of substances will be in accordance with best practice guidelines on maximum volumes and frequencies such as those in Wolfensohn and Lloyd (2013) Handbook of laboratory animal management and welfare. 4th Edition. Chichester: John Wiley and Sons. Surgical procedures will be performed in accordance with LASA best practice guidelines on aseptic surgery.

All animals are group-housed in filter top cages within ventilated cabinets (Scantainers) with enrichment such as tunnels, chew sticks and nesting material

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date by reading scientific articles, attending conferences and animal welfare meetings. I will also keep in touch with the named veterinary surgeon and named animal care and welfare officer. We will consult the Named Veterinary Surgeon, Named Animal Care and Welfare Officer and Named Information Officer on improving the experimental design.

148. Role of tumour microenvironment in 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, metastasis, tumour microenvironment, immune cells, immunotherapy

Animal types	Life stages
Mice	adult, pregnant, neonate, embryo, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify new (immuno-)therapeutic targets in metastatic cancers.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Solid cancers, including breast and bowel cancer, that are of main interest for our lab, represent a main cause of death worldwide. Both breast and bowel cancer are among the top 4 more prevalent cancers, still associated with high mortality rates. Current cancer mortality rates are higher than 30/100.000 for breast cancer in female and bowel cancer in males. Indeed, breast cancer, the most common cancer in women in UK, accounts for around 55,500 new cases and around 11,500 breast cancer deaths in the UK every year. Bowel cancer is responsible for around 16,600 deaths, and around 42,900 new people are diagnosed with bowel cancer in the UK every year. The economic costs associated with cancer are obviously huge, and total economic cost of cancer in UK only has been

estimated to be £7.6 billion in lost wages and benefits per year in 2020. A report in 2020 revealed that bowel cancer only costs the UK economy more than £1.7 billion a year. Costs for treatments are also massively impacting the NHS. Chemotherapy costs the NHS an estimated £1.4 billion a year (over £248 million for breast cancer chemotherapy only).

Notably, the treatment of cancer at the primary site is effective in most cancers, and particularly in breast cancer. Unfortunately, treating metastatic cancer remains a huge challenge. Indeed, metastasis represent the main cause of death in cancer and account for more than 90% of cancer-related deaths, highlighting the urgent need for novel therapeutic strategies able to effectively target metastasis.

A better understanding of the basic biology of metastasis is essential to develop new treatments. This study aims to study how different components of the tumour microenvironment (TME) co-operate to create a tumour-supportive environment, and how these tumour-promoting mechanisms can be inhibited to ultimately impair metastasis. A particular focus of this study will be the immune cells, how they support metastatic colonisation and how this support can be impaired to prevent metastasis or stop metastatic growth.

The ultimate goal of our work is the identification of novel and more effective immunotherapeutic targets to benefit a larger cohort of cancer patients, and particularly patients at risk of developing metastasis.

What outputs do you think you will see at the end of this project?

This research programme aims to better understand the interactions occurring between the cells in the tumour microenvironment and the cancer cells. In particular we will focus at the interactions occurring at different stages of metastasis to support metastatic growth and how these interactions can be impaired to prevent metastatic disease or stop metastatic growth.

This research aims to identify new therapeutic targets that will then be tested more in depth in other pre- clinical and clinical studies.

The data generated will be presented at scientific meetings and included in peer-reviewed publications.

Who or what will benefit from these outputs, and how?

I expect that the research outputs generated with this research will lead to scientific publications and therefore be shared to benefit the whole scientific community by the end of this 5-year research programme.

Moreover, the experimental data generated will be routinely presented and discussed at Departmental meetings in the Institute during these 5 years, as well as presented at Cancer conferences. This will help fostering scientific collaborations to accelerate the generation of new knowledge in the field.

How will you look to maximise the outputs of this work?

I have already started several collaborations to help maximising the impact of our work in cancer research, both within the Institute as well as other research Centres in UK.

Moreover, the generation of new scientific data and the possibility to present them at local as well as international conferences will help to integrate our knowledge with others' and get mutual benefits for the progress of our investigation.

Species and numbers of animals expected to be used

- Mice: 8100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We aim to study cancer initiation and progression, with a particular focus on metastasis, the spread of cancer from the primary sites to distant organ. While treatment of cancer at the primary sites has significantly improved life expectancy in patients in most cancer, metastasis still remains largely uncurable, accounting for more than 90% of death in cancer.

A particular focus of this study will be the immune cells within the tumour microenvironment, with the ultimate goal of identifying new immunotherapeutic targets in metastasis.

To study a systemic process involving multiple organs, such as metastatic cancer, a living organism is needed. We will use mice as a model of disease onset and progression as these are the smallest living creatures that can faithfully mimic what is happening in human disease.

We will mainly use adult mice as they will have a fully developed immune system, one of the key features that a tumour 'hijacks' in order to grow and spread.

We will also use mice that have been genetically engineered to develop cancer. The types of mutations introduced into the mice are usually designed to mimic human disease. This is a key requirement to obtain scientific results that will ultimately benefit cancer patients.

We also aim to use in this study inducible genetic mice, where genetic changes can be induced after the administration of a drug. This will allow to switch on or off specific genes that may play a key role in cancer initiation and/or progression.

Typically, what will be done to an animal used in your project?

Breeding: genetically modified mice will be bred under this licence to study the effect of specific molecules in cancer disease. This will include tumour mouse models.

Breeding: genetically modified mice will be bred to study the role of specific molecules in cancer disease. We will also bread tumour-bearing mouse models. Mostly we will be using models where heterozygous females spontaneously develop tumours in the breast. These tumour-bearing animals will be mostly used for tissue collection.

A minority of genetically modified tumour-bearing mice will be administered treatments in order to impair tumour initiation and/or progression.

To generate tumour growth at the primary site, we will also be administering cancer cells to the mice, typically by injecting cells subcutaneously or in the mammary gland once. Occasionally one injection on each flank may be required. These procedures will be done under general anaesthesia. As I have refined injections in the mammary fat-pad without need to expose the mammary gland, we do not expect these administration routes, subcutaneous or fat-pad, to require surgery.

We will also inject cancer cells via a route deemed to promote metastasis, most commonly intravenous injection. Intrasplenic injections to study liver metastasis will also be used. These will be done under anaesthesia. More commonly this procedure will be carried out using a non-surgical image- guided injection of cells into the spleen. Some animals in b), c) and d) will also receive therapy by mouth or by injection and will be monitored for any side effects. These animals may also be monitored by in vivo imaging for disease regression/progression. Animals will be anaesthetised using inhalation anaesthesia for minimally-invasive imaging (typically 30 min - 1 h duration) Most experiments will be finished within a month from the start of the treatment, when the animals will be humanely culled and their tissues collected for analysis.

Treatments, including the use of therapeutic drugs may be administered at any stage via different routes to animals. Doses know not to cause adverse effect to the animals will be used. Where a drug and/or dose is not known we will use a small pilot study to determine a dose that is both well tolerated in the mice while still showing effective results that will support objective.

I have started to use mouse models to study metastatic cancers in 2012 and I have direct experience in using most of the cellular cancer models that I am proposing in this application. I am therefore familiar with the timings of disease progression. In experimental cases where timelines of metastatic lesion appearance are unknown, animals will be closely inspected for any clinical signs or symptoms of disease. Manual health monitoring at this stage may be complemented by imaging modalities where appropriate.

When studying metastasis, in most cases we will focus on early metastasis. Therefore, animals will be usually culled at a stage when they have micro-metastases. At this stage we do not expect the mice to experience any discomfort due to the metastatic growth.

Aged mice: in a small number of experiment (<10% of mice in Protocol 5), aged animals (older than 15 months) may be used. Tumour growth will be induced in this mice by cancer cell administration, typically via intravenous injection, to study lung metastasis. Administration of cancer cells via subcutaneous or orthotopic injections in the mammary gland may also be used.

What are the expected impacts and/or adverse effects for the animals during your project?
No harm or adverse effect is expected from breeding.

Animals should experience no more than transient discomfort due to injections or imaging/anaesthesia. Anaesthesia times will be kept to minimum requirements to ensure fast recovery. Tumour growth and metastasis may cause long lasting mild or short term moderate discomfort including weight loss and short term pain but animals will undergo

daily welfare checks for clinical signs and will be humanely culled to prevent any effects beyond moderate.

In the experiments with aged animals we will use more specific monitoring for aging mice (Wilkinson MJ, et al. Progressing the care, husbandry and management of ageing mice used in scientific studies. Laboratory Animals 2020 54:225-238). Importantly, aged animals will not experience higher degree of severity. More details will be given in Protocol 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 animal type)?

All animals are expected to experience mild-to-moderate severities, mainly due to tumour growth at the primary or metastatic site. Mice that will be used for breeding in Protocol 1 are expected to experience only mild severity due to tissue biopsy needed for genotyping.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are essential for studying complex diseases such as cancer; and a requirement for testing pre-clinical and promising scientific therapies that we would hope to translate into patients.

This work in particular aims to study the immune cell response during the metastatic process, in order to understand how the tumour microenvironment supports metastatic initiation and progression, and how this support can be blocked to impair metastasis.

Since metastasis is a systemic disease, involving the entire organism, living animals are required. I will be using mice, as animal model. Mice have a short life span, fast reproductive rate, known genetic background, close genetic and physiological similarities to humans, and recapitulate many aspects of the genesis, progression and clinical course of human cancers. Of particular relevance for this work, extensive literature supports the value of using mice as model system for immunological studies in cancer, thanks to their ability to recapitulate many features of the human immune system. Indeed, research on mice has hugely contributed to our knowledge on cancer and immunology.

Which non-animal alternatives did you consider for use in this project?

We have previously optimised a 3D co-culture system on a scaffold to mimic the metastatic environment in the lung and study inter-cellular interactions between three different cellular components. This system will be instrumental to validate in vitro the role

of molecules influencing the inter-cellular cross-talk that will be identified by in silico analysis.

Moreover, we are currently establishing more 3D in vitro co-cultures and assays, by mainly using tumour spheroids, to assess how the perturbation of inter-cellular interaction affects cancer cell proliferation and invasion in vitro.

Also, in collaboration with other research groups in the Institute, to help answering some of our questions about immune-stromal interactions in cancer, we will be using de-cellularised tissue models they have been recently established.

Why were they not suitable?

In vitro systems to study inter-cellular interactions in cancer have been established and some relevant to our studies (eg. heterotypic 3D co-cultures) will be certainly used.

However, since metastatic cancer is a systemic disease involving multiple organs, these in vitro systems have major limitations when studying the role of the tumour microenvironment. Indeed, these in vitro platforms do not entirely recapitulate the complex and dynamic interactions occurring in cancer, and particularly in metastasis.

This is particularly relevant when studying the role of immune cells, including immune cells derived from the bone marrow and recruited to the tumour site, is needed. The in vitro systems are certainly valuable and we will also use some to refine and reduce the number of mice required in this study. However, the validation of in vitro findings to assess their relevance in metastasis is still necessary to draw biologically relevant conclusions and strongly support the anti- metastatic potential of the new identified therapeutic factors for future 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?

Animal numbers in experimental groups are estimates based on the size of the effect that we expect to see between our various test conditions, standard agents and controls (using our own previous data, pilot data and/or that seen in the literature) and individual variability within groups, and will be different for each experiment.

I have based our estimates of animal numbers on previous studies I have performed during my post- doctoral studies where I have used most of the experimental setting I am proposing to use in this application.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experiments will be carried out following good laboratory practice. All experiments will be designed after careful examination of the literature, with regards to treatment i.e. drug dosage, treatment regime and with the aid of the NC3R's Experimental Design Assistant. Whenever possible use of in vitro assays will reduce the numbers of animal required. Notably, I have previously set-up a 3D co-culture system on a scaffold to mimic the metastatic environment in the lung. Moreover, we are currently optimising in vitro assays by using co-cultures with tumour-spheroids and we are planning to develop similar platforms by using human cancer cell lines and immune cells isolated from human blood.

The combination of these approaches will reduce the number of animals required in our studies, by having the possibility to perform valuable preliminary screening in vitro.

Ultimately, the potential therapeutic targets we will identify in our studies will needed to be tested in vivo. However, the minimum number of animals will be utilised for each experiment performed under this Programme of work while maintaining a reliable and measurable output.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most of the experimental setting I will be using in this study have been previously optimised during my post-doctoral studies.

In case new setting, as well as cellular models we have not previously used, are needed. We will refer to existing literature to design small pilot experiments to identify the most suitable setting (i.e. the number of cells to inject, the number of days needed to grow micro-metastasis, etc) for our experimental needs.

To ensure reproducibility of results, standard operating procedures are usually followed to minimize confounding variables such as using animals of the same genetic strain, of similar weight and age. Also, similar housing conditions will be ensured. Importantly, mice will be randomly assigned to different experimental groups, and researchers blinded to test treatment allocations.

Where high skill is required for a procedure (eg. echo-guided intrasplenic injections) we will plan to have the same personnel performing it in the different experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Spontaneous tumour development models - These mice will naturally develop breast cancer so that we can investigate the biology of the developing disease and test our treatments in a model that most closely matches the human disease. Usually these mice

do not develop any pain or discomfort as a consequence of their developing cancers and we will cull the animals before such discomfort occurs due to tumour size.

Metastatic colonisation model - Intravenous or intrasplenic injections will be used to seed the cancer cells at the metastatic sites, in order to study metastatic colonisation and progression. In most cases we are going to study early metastasis and will cull the animals when they have micro-metastases. At this stage we do not expect the mice to experience any discomfort due to the metastatic growth. When case mice growing metastases at later stages are needed, we will terminate the experiment at a time-point where mice are not showing any clinical signs of pain due to the cancer growth. However, the mice will be closely monitored daily and they will be culled if they present clinical signs due to the tumour growth.

Syngeneic tumour implantation (subcutaneous and orthotopic) - Cancer cells will be transplanted to generate primary cancer growth. Orthotopic cancer implants are within the organ of cancer cell origin, while subcutaneous injection of cancer cells will be performed on the side flanks of the mice. I have refined mammary procedure to inject without need for surgical incision. In longer term studies, mice will be monitored with ultrasound or MRI imaging to measure cancer growth. Immunocompromised models - Immunocompromised mouse models will be used to help differentiating the contribution of the innate immune system to the tumour initiation and progression. These animals will be housed in individually ventilated cages to limit pathogen exposure and subclinical disease.

Why can’t you use animals that are less sentient?

To study the dynamics of a systemic disease such as metastatic cancer, and how the disease progression can be altered by using therapies, in vivo models are still necessary.

We will be using mouse models, as less sentient animals often diverge from their similarity to humans genetically. Moreover, as this study aims to identify new immune-therapeutic target, mouse models will be extremely valuable. The choice of mice as model organism is well-supported by literature showing their value as model system for immunological studies in cancer, thanks to their ability to recapitulate many features of the human immune system. Indeed, research on mice has hugely contributed to the development of current immunotherapies that are benefitting millions of cancer patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To limit severity in our cancer models we will:

measure tumours regularly to prevent tumours exceeding the mmaximum authorised size and close monitoring for tumour ulceration and /or impact on nearby tissue that could impaired animal wellbeing and /or long term pain.

determine humane endpoints to minimise severity beyond moderate clinical signs while delivering valid scientific outcomes

use fluorescently labelled cells to image mice for tumour spread and metastasis, as a valuable refinement and reduction approach.

perform pilot studies on orthotopic cancer models when needed, to characterise kinetics of tumour growth and metastasis.

Mice growing a primary tumour as well as mice who undergo experimental procedures such as surgery or injection of tumour cells to generate metastasis, are monitored daily for any adverse effects to avoid suffering at all times.

Notably, I have started working with mouse models of metastatic cancers in 2012 and have extensive experience of working with most of the models I am proposing to use in this application, and I am aware of health problems the mice could develop and can take timely action to minimise suffering, to ensure pain relief is quickly administered whenever necessary. Moreover, we have clear guidelines on humane endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have been following the ARRIVE guidelines when planning the proposed protocols and will follow the most up to date version (ARRIVE guidelines 2.0) published by the NC3Rs.

We will also make use of the Experimental Design Tool (https://eda.nc3rs.org.uk).

Attalla S, et al. Insights from transgenic mouse models of PyMT-induced breast cancer: recapitulating human breast cancer progression in vivo. Oncogene 2021 40:475-491. This work gives clear indication on the MMTV-PyMT mouse model, the main spontaneous model of metastatic breast cancer we will be using.

Morton DB, et al. Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Laboratory Animals 2001 35:1-41. This work’s guidelines will be closely followed when the administration of substances is required.

Wilkinson MJ, et al. Progressing the care, husbandry and management of ageing mice used in scientific studies. Laboratory Animals 2020 54:225-238. This work gives specific guidelines when working with ageing mice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our team will be using the NC3R's website as a source of current guidelines. In addition, our on-site Veterinarian typically informs all licence holders of new advances in the 3R's. Moreover, we will benefit of an Animal Technician Service on site that will suggest amendments to Procedures, for the benefit of the 3R's.

149. Neural correlates of motor control and their application to Brain-Computer Interfaces

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

brain, movement, neurons, neuroprosthetics, brain-computer interfaces

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how the brain controls a wide range of skilled movements. In parallel, we will apply our findings to develop a novel “brain-computer interface” that enables users to mentally control an external device more naturally.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Movement is how we interact with the world and others. A variety of diseases and neurological injuries partially or completely impair our ability to move. These conditions are very prevalent: as many as one in four people over fifty years of age suffer from a movement disorder. Brain-computer interfaces (BCIs) that control external devices by "reading out" the user’s intent from brain recordings hold great promise to help this large group of patients. But improving these BCIs so they can become widely used in clinics requires improving our understanding of how the brain works. The proposed research will help us understand how different parts of the brain work together to cause movement. In

parallel, we will combine these findings with technological advances to develop BCIs that are more intuitive to control.

What outputs do you think you will see at the end of this project?

The goal of this project is twofold: 1) to learn about how different parts of the brain work together to control movement; 2) to apply these findings to explore new ways of “reading out” someone’s intent from brain recordings and use them in new “brain-computer interfaces” that are easier to control.

As part of our first goal, we will generate new information in the form of datasets and mathematical analyses that will be published in scientific journals and discussed at scientific conferences. These findings will provide better understanding of how groups of neurons from different regions of the brain learn and control our movements.

As part of our second goal, we will provide a comparison of how well a subject’s intended action can be detected from these different regions of the brain. These results, which we will also publish in scientific journals and present at conferences, will inform the design of future "brain-computer interfaces" that enable paralysed individuals to control devices using their thoughts.

Who or what will benefit from these outputs, and how?

In the short term, the scientific community will benefit from the new data and knowledge on how the brain controls movement we will produce. We will seek to further the impact of our work by making the data available to both current and new collaborators.

In the medium term, these results may be useful for the growing number of companies developing on “brain-computer interfaces”, devices that allow paralysed individuals to control computers or robots with their thoughts. These companies may use our results to identify new brain regions to record from and improve the performance of their products. This way, our results could enable better technologies that improve the lives of people with conditions such as stroke or spinal cord injury.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in preprint servers and open- access journals, and through presentations at scientific conferences and meetings. Experimental data will be shared with collaborators.

Species and numbers of animals expected to be used

- Mice: 2100
- Rats: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We require the use of an animal model to achieve our two goals: 1) understanding how the brain controls movement and 2) developing a novel “brain-computer interface” that enables (paralysed) users to control an external device with their “thoughts” in a more natural way. We will be using adult rats and mice as both their behaviour and the organisation of their brains are similar to ours, while being a low order animal. Any non-protected animal alternative would be an inadequate model because their brains are too different from the human brain to be able to translate our findings.

Typically, what will be done to an animal used in your project?

During a 1–2 h surgery, we will implant a device that allows "connecting" a mouse to a system that records brain activity during movement. Afterwards, we will train the animal over several experimental sessions to perform a task (typically, daily over one or two weeks depending on the complexity of the task). Our tasks, which are designed to understand how the brain controls movement, include running on a treadmill either spontaneously or following sounds or pleasant odours, as well as picking up treats or using a joystick.

Following this training period, we will carry out a second 1–2 h surgery to make a small window in the skull to gain access to the brain. These mice will then undergo experiments in which we will record brain activity during movement, to understand the relationship between the two. Brain recordings will be typically performed with very fine electrodes that are inserted at the beginning of the session and removed at the end, without causing any harm to the animal, following well-established practices in the field. In some days, mice will control a "brain-computer interface" using these same recordings, e.g., to "mentally" regulate the frequency of a sound and bring it to a target value to receive a reward. The combined duration of these experiments, which will be carried out five times a week, will typically be under one month.

Some of these experiments will require mice to perform many repetitions of a laboratory task, for example to study how a skill is developed over time. To get mice to practice a task many times, we will have to limit their access to fluids while they are in their cage: instead, mice will get a fluid reward after each successful trial in the laboratory. We will ensure that each mouse receives the appropriate amount of fluid every day by giving them an extra volume to achieve a healthy minimum, when necessary.

Answering some of our scientific questions does not need recordings during behaviour. In this case, we will perform brain recordings under anaesthesia from which the animals will not wake up, to minimise suffering.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will have two surgeries to implant a device that allows "connecting" them to the recording system, and to make a small window in the skull, respectively. They are expected to recover quickly and will be given painkillers and post-operative care just like people recovering in hospital.

Neither the behavioural experiments nor the brain recordings will lead to any negative effects. In fact, in order for us to study how the brain controls movement, we need mice to engage in behavioural tasks, which they only do if they are healthy and feel comfortable in the laboratory.

As mentioned above, a subset of all mice will undergo fluid control to get them to practice a given task repeatedly in the laboratory, which is crucial for us to understand how skills are learnt. This is a well- established approach in the field and, even if animals lose a bit of weight early in training, they adjust rapidly to this schedule and go back to their normal weight usually within a week. In the extremely rare occasions in which mice do not recover their weight, they will be culled.

Animals only participating in experiments under anaesthesia will be culled without regaining consciousness and thus will not suffer any adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 95 % moderate suffering, due to the implants for behavioural and brain recording. Note that mice will be closely monitored and provided with pain relief before and after surgery, and will be supervised by trained individuals.

Rats: 5 % non-recovery (they will undergo terminal experiments under anaesthesia, so they will not suffer any amount of pain).100 % non-recovery (they will undergo terminal experiments under anaesthesia, so they will not suffer any amount of pain).

All animals will be humanely killed at the end of an experiment.

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 require the use of an animal model to understand the complex and unknown ways by which the brain controls movement. Any non-protected animal alternative would be an inadequate model because they do not have a brain that is similar enough to ours.

Which non-animal alternatives did you consider for use in this project?

We have considered the following alternative approaches:

- computer modelling,
- cell cultures and brain slices, and
- non-protected species.

Why were they not suitable?

Computer modelling: Where possible, we will use computational models to test hypotheses before doing experiments on animals. However, scientists do not know enough yet to simulate the entire brain and how it moves our body to answer the questions in this project only using models.

Cell cultures and brain slices: Understanding how the brain controls movement requires recording brain activity during behaviour, to identify the relationship between the two. This cannot be achieved in cell cultures or brain slices since they are not connected to a body.

Non-protected species: These species have brains that are very different from the human brain, and perceive the world and move within it in ways that are very different from ours. Thus, results obtained from non-protected species cannot be used to understand the human brain, which is our ultimate goal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 projected number of animals reflect the number necessary to achieve the scientific objectives outlined in the programme of work described in this application. We have estimated the number of mice and rats based on the anticipated numbers of experiments, the numbers of experimental groups, and the numbers of animals in each group. These estimates are based on the standard number of animals used to show big effects in our field.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We carefully plan all our experiments to minimise the number of animals we need to use to get the most information possible. In many cases, we will record brain activity from the same animal during two phases of a study. This will allow us to maximise the data generated by one animal, thus reducing the overall number of animals needed without increasing the amount of suffering of each individual animal. All experiments will be designed in such a way as to be publishable under the NC3Rs ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will take all reasonable steps to reduce the number of animals used in our project. We will collect as much information as possible from every animal, for example, by making many measurements over different experimental sessions. This will often include the same animal taking part in two phases of a study that are aimed at answering interrelated scientific questions. We will use consistent experimental techniques across studies, thus reducing 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice and rats are the most suitable models for this type of research because they share close resemblance to human biology, and the way they perceive the world and interact with it is relatively similar to ours. Due to experimental and technical considerations, mice will be used in all the behavioural experiments, and in some experiments under anaesthesia. A subset of the studies under anaesthesia will be performed in rats, given that their bigger brains and bodies make it easier to monitor many areas of their nervous system simultaneously, which will allow us to map the sensorimotor "networks" of the brain more extensively.

All surgeries will be performed during anaesthesia. During these procedures, we will test for effective anaesthesia by assessing the toe pinch reflex, and will administer additional anaesthesia if necessary. Many of our experiments require simultaneously measuring brain activity and behaviour; during them, animals will be closely monitored by trained individuals. Some of the questions we will investigate require that the animals perform many trials during each experimental session. To achieve this, we will restrict their access to water between experiments, and provide liquid rewards after each successful trial. Importantly, we will always ensure that the animals receive at least a daily minimum amount of water that keeps them in good health. Whenever possible, we will perform studies under terminal anaesthesia, to minimise harm to animals.

Overall, animals will be constantly supervised by trained individuals and advice will be sought from the veterinary team if there is any cause for concern. We will use anaesthesia, analgesia, and humane endpoints to limit suffering.

Why can’t you use animals that are less sentient?

Less sentient animals are further apart from human biology. In contrast, mice and rats are also mammals and have brains, nerves, muscles, joints, and sensory receptors that are similar to ours. The way they experience the world and behave are also quite close to the way humans do. We need to work with animals that are fully grown both from a practical perspective and to be able to translate our results to adult humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinement measures carried out in this project will include use of anaesthesia and pain-relief medication as well as enrichment of the animal's environment. We will frequently monitor animals, especially after they have received implants, and will increase monitoring

if any adverse effect manifests; depending on the severity of harm, the animal will be treated following a veterinarian's instructions or humanely killed.

Animals taking part in behavioural experiments will first be accustomed to people, to reduce their stress levels. During any such 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.
Throughout 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 you follow to ensure experiments are conducted in the most refined way?

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, as well as the LASA guidelines for aseptic surgery and administration of substances.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep informed about any advances by reading scientific publications and speaking with colleagues within our own university and elsewhere, including not only other scientists but those directly involved in the care and welfare of animals. We will constantly review our protocols and experimental design to reduce, refine and replace the use of animals. We will also attend the frequent meetings held at our institution on the advances in the 3Rs and their implementation.

150. Oxidative stress and inflammation in retinal degeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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
mouse, retinal degeneration, oxidative stress, inflammation, therapy

Animal types	Life stages
Mice	adult, juvenile, aged, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the project is to use mouse models for understanding the development of disease (called pathogenesis) in the following conditions: diabetic retinopathy, age related macular degeneration and retinitis pigmentosa (RP), these are major types of retinal degeneration.

Retinal degeneration refers to retinal cell loss and is a major cause of blindness in the world.

By understanding the diseases, we can develop potential therapeutic strategies for these conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

Retinal degeneration refers to retinal cell loss and is a major cause of blindness in the world. Diabetic retinopathy (DR), age related macular degeneration (AMD) and retinitis pigmentosa (RP) are major types of retinal degeneration. The disease mechanisms of these conditions are not fully understood and there is also no cure for these conditions.

DR is one of the most common complications of diabetes and the leading cause of visual impairment and blindness in the world, affecting 90% of patients with over 20 years duration of diabetes.

AMD is the commonest cause of visual impairment in the developed world.

It is important to undertake this work because oxidative stress and inflammation have been linked to retinal degeneration. By studying the role of oxidative stress and inflammation in mouse retinal degeneration models we hope to generate new insights into the molecular mechanisms underlying the development of retinal degeneration, which will lead to the identification of potential treatments.

What outputs do you think you will see at the end of this project?

The main outputs at the end of this project are expected to be new insights into the functional role of oxidative stress and inflammation in the pathogenesis of retinal degeneration. This may include the identification of novel biomarkers of disease or new drug targets. We will share this with the scientific community via peer-reviewed publications in international scientific journals and presentations in national and international conferences.

Who or what will benefit from these outputs, and how?

The main beneficiaries of the outputs generated in the period of this licence will be other scientists investigating the disease mechanisms of retinal degeneration. The data we report will provide new ideas and insights to advance their studies. The research will also benefit clinical researchers who are investigating the underlying causes of retinal disorders in patients. In addition, our research will also provide important new insights that may advance the work of others in this general area. Our work may also impact on researchers working in the pharmaceutical industry through the identification of potential new biomarkers (i.e. measurable indicators of some biological state of condition) and drug targets that can be exploited to aid disease diagnosis and treatment. There is also likely to be an impact on the third sector as we will seek to interact with relevant charities, such as Fight for Sight and Sight Research UK, as ourwork progresses.

How will you look to maximise the outputs of this work?

The outputs from this work will be maximised through a range of collaborations with other colleagues in the UK and overseas. We will collaborate with experts in a range of omics technologies (these are technologies used to explore the roles, relationships, and actions of the various types of molecules/signal pathways involved in the pathogenesis of retinal degeneration) to ensure that we develop a detailed understanding of molecular changes that are caused by deficits in disease-causing genes/proteins, which may be linked to retinal degeneration.
We will also attend relevant national and international conferences to present the results of our work as it progresses-this will ensure a constant influx of new ideas and scientific critique. This will be complemented by delivery of invited seminars at other institutions and attendance at general scientific meetings to ensure we are interacting with a broad range

of basic, translational, clinical and industrial colleagues. These fora provide an excellent opportunity to also discuss unsuccessful approaches, unexpected findings etc and to limit any duplication of effort. We also interact regularly with colleagues from other disciplines e.g. chemistry and seek to develop new or modified small chemicals to maximise our scientific endeavours.

Collectively, these approaches will ensure that any publications are of the highest quality and impact. Collectively, these approaches will ensure that any publications are of the highest quality and impact.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mouse models to investigate the underlying molecular pathology of retinal degeneration. The primary reason for using mice is the ease of genetic manipulation of this species and the ability to obtain relevant mouse models from appropriate repositories. In addition, there is an extensive literature using this species to investigate underlying causes of retinal disorders. Indeed, the main mouse models used in this work have been shown by our group and others to exhibit many of the key features seen in patients with retinal degeneration. Mice are typically used from three months of age till 15 months.

Typically, what will be done to an animal used in your project?

Typically, genetically altered (GA) or chemically-induced retinal disease mice are culled in a humane schedule 1 procedure and eye samples will be removed for analysis.

Some mice will receive drugs that cause damage to the pancreas and they become diabetic.

In addition, some mice are given treatments designed to improve the condition of the animal either in the diet, through a tube leading down to the stomach (gavage), eye drops, into the peritoneal cavity (intraperitoneal), intravenous or under the skin (subcutaneous) administration that may run for a duration of six weeks (depending on the route).

After treatment, some mice are used in a range of behavioural tests that may run for a duration of a few days. After behavioural/physiological measurements are completed, mice will be humanly culled and tissues will be taken for furtheranalysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The volume and frequency of injections will be used according to local good practice guidelines.

Diet manipulation: some mice may initially show reduced food intake of the altered diet, however, with time their intake increases. To minimise this adverse effect, the introduction of the new diet will be made gradually. Animals are expected to develop high lipid levels in blood. This in turn may increase the risk of atherosclerosis and, in its wake, myocardial infarction, liver failure, stroke, pulmonary embolism.
However, in our experience, these adverse events are very rare (less than 1 in 100 animals). If any animal develops signs indicative of any of the aforementioned syndromes, it will be removed from the study and immediately culled.

Induction of diabetes: we inject a substance that will result in the destruction of pancreatic islet β-cells and the development of diabetic signs including increased drinking of water, frequent urination and reduced weight gain. The impact of these disturbances will be minimised by ensuring access to water at all times and provision of plentiful bedding (changed as often as necessary). Regular monitoring of weight will be carried out. Some animals may develop cataracts, however, this should not prevent them from accessing water or food. If any animal reaches 20% loss of its initial body weight, it will be humanely killed.

Treatment: Mice will be carefully monitored. We predict that the administration of small molecules, gene cells or natural products will decrease the severity of pathology in the treated mice. If there are any potential side effects (i.e. toxicity) caused by those small molecules or natural products, any animal displaying weight loss of 20%, and/or displaying signs of distress, piloerection and hunched posture will be humanely killed.

When work under terminal anaesthesia in involved, the level of anaesthesia will be maintained at sufficient depth for animal to feel no pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most (estimated 80%) expected to be mild; some (estimated 20%) expected to be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

It is not feasible to produce an adequate model for a functional retina in vitro, and therefore we need to use living models. Animal usage is essential to allow us to examine the effects of disrupting specific genes or dysfunction of physiology on development and progression of retinal degeneration.
Genetically-altered mouse strains are the most frequently used animal model to study the importance of specific genes/proteins for (patho)physiology and to provide appropriate

disease models, and their use in the current project is essential. Where possible, we will complement these studies using cell lines- these are cells that have been maintained in culture for an extended period of time and are not taken from currently live animals. However, the use of animals is essential to achieve the aims of our investigations.

Which non-animal alternatives did you consider for use in this project?

I have used in vitro mammalian cell systems to characterise the functional role of oxidative stress and inflammation. I also have used stem cell model to characterise retinal disease gene function. We will continue to use mammalian cell lines wherever possible to limit animal usage. However, in vitro assays cannot adequately characterise the effects of oxidative damage and consequent inflammation.

Why were they not suitable?

Whilst analysis of cell lines can (and will) provide important complementary information, it is not possible to study higher integrated processes such as visual function using cell-based assays, and simple model organisms (e.g. yeast) lack the required complexity and physiological pathways to allow a detailed understanding of functional roles of oxidative stress and inflammation that can be translated to human physiology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers have been estimated based on usage of mouse models over the previous five-year period. I anticipate that similar numbers of these mice will be used over the 5-year period of the project licence (~700 per year).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The proposed methods, experimental designs and methods of analysis of the results have been discussed with statisticians. The design of individual experiments will generally involve factorial designs, which maximise the information obtained from the minimum number of replicates. We will use the least number of animals to provide an adequate description, generally on the basis of previous experience (ours, or from the literature). In terms of the numbers of animals required, based on our previous work we expect 8 mice per analysis/treatment group should be sufficient to obtain the required results (e.g. statistical significance with one-way or two-way analysis of variance, t-test). Clearly, the exact numbers of animals required will vary with the particular experimental design, the estimate of the coefficient of variation, and so on. For the qualitative experiments, the amount of material required will be the minimum necessary to provide an adequate description.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use the same animals in behavioural tests and physiological measurements where this can be achieved without causing undue stress to the animals and without adversely affecting the outcome of experiments. Similarly, we will take any physiological measurements and tissue samples from the same animals, where this is appropriate. We will also carefully monitor all breeding animals to ensure that breeding is timed with the requirement for offspring. These steps will limit/optimise the number of animals used in this project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered (specific gene disrupted or transgenic gene) and chemically-induced retinal disease models that we have been studying for the last five years. These mice show no obvious clinical signs or reduction in life-span. The experiments we are undertaking are either minimally invasive physiological measurement or extraction of tissue following schedule 1 killing.

These methodologies for this project are designed to minimise any distress to the animals. Some animals may receive treatment with small chemicals or natural products. Animals will be humanely killed at the end of the experiments.

Why can’t you use animals that are less sentient?

Analysis of complex visual function and analysis of development and progression of retinal diseases cannot be done using less sentient species. The mouse has been commonly used to study the pathogenesis of retinal diseases, the phenotypes of mouse retinal diseases reflect the pathologies seen in man.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

This is a project licence with mild or moderate severity banding, but nevertheless we will seek to implement refinements wherever possible, e.g. ensuing our breeders are at peak age for mating, that they do not have too many litters, that genotyping methods (if required) are the mildest possible.
Animals under study will be closely monitored and housed in social groups with regular health checks. We will use the least invasive route of injection whenever possible; Early end points will be used when scientific goals have been achieved.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs EDA and breeding information.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Information from the NIO and review the NC3R website.

151. Mechanisms of mammalian birth defects and inherited metabolic disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Congenital anomalies, embryonic development, neural tube defects, inborn errors of metabolism, therapy

Animal types	Life stages
Mice	adult, pregnant, neonate, juvenile, embryo, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project uses wild-type and genetic mutant mouse strains to determine the processes in the embryo, fetus and post-natal individual that cause congenital anomalies (birth defects) and inherited disorders of development. The work focuses primarily on neural tube defects (NTDs), other nervous system malformations and inherited metabolic disorders to improve the understanding these disorders and develop new clinical therapies for 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?

Approximately, 1 in 20 babies, equating to 8 million births per year are born with severe anatomical malformations (also known as congenital anomalies or birth defects). These anomalies lead to approximately 300,000 deaths each year and are the leading cause of infant mortality in developed countries. Many children with congenital anomalies have physical and/or mental disability and require life-long medical treatment. Considering Neural Tube Defects (NTDs) alone, at least 250,000 pregnancies are affected by this crippling birth defect per year, world-wide. Children with spina bifida are often paralysed from the waist down, lack sensation in the legs, have vertebral deformities, hydrocephalus (water on the brain) and frequent kidney abnormalities. Individuals and their families with birth defects of this type have many challenges to cope with. Moreover, healthcare services incur very significant financial cost in caring for children with birth defects. Unlike most other causes of childhood mortality, congenital malformations have shown no reduction, and are even increasing in prevalence worldwide.

While secondary preventive methods (e.g. termination of pregnancy after prenatal diagnosis) have resulted a reduction in the frequency of children born with severe birth defects in some countries, a key goal is primary prevention. That is, to be able to normalise the development of fetuses with a predisposition to birth defects, enabling the birth of a healthy or minimally disabled child, and so avoid damaging effects in postnatal life. One example in clinical practice is the use of folic acid supplementation, which has significantly reduced the frequency of NTDs. However, it is unlikely that this public health intervention will eradicate NTDs, as many cases appear folic acid-resistant. Hence, new methods are needed to enable more NTDs to be prevented, and to extend primary prevention to other malformations for which there are currently no therapies available. Other disorders to be studied in this project are linked to alteration in Folate One-Carbon Metabolism (FOCM), a network of biochemical reactions that is needed in almost every cell type in the body.
FOCM is associated not only with birth defects such as NTDs but inborn errors of metabolism and other human diseases including cancers, liver disease and age-related cognitive impairment. In this project we focus on the impact of FOCM in NTDs (both as a risk factor and preventive therapy), hydrocephalus and inherited life-limiting metabolic disorders such as Non-Ketotic Hyperglycinemia (NKH).

Optimising preventive approaches for congenital anomalies requires a better understanding of their underlying causes, both genetic and mechanistic. However, understanding the causes of these anomalies in humans is challenging owing to their complex genetics and the possible contribution of non-genetic factors such as diet and environment. Hence, despite the massive accumulation of human genomics data it is not yet possible to explain the cause of anomalies in most individuals. As one means to address this gap in knowledge it is necessary to use mouse models as one of the tools to assess whether identified genetic variants are causal.

What outputs do you think you will see at the end of this project?

The present research programme uses the following genomics advances, to investigate the origin, development and prevention of birth defects:

Gene sequences as tools for identifying where and when a given gene is active in the embryo; Gene targeting methods for creating new mouse strains that either: Lack a given gene function (knockout strain) or Target loss or gain of function of a given gene in a time-dependent or tissue-specific manner (floxed alleles and Cre driver lines); New information

to be generated (and published with open access) will include: Identifying the genetic and environmental basis of congenital anomalies
We and others have identified a large number of genes for NTDs in mice of which some have also proven important in humans. Multi-gene interactions are likely to underlie most NTDs in humans, and have been studied so far only in mice. Altered folate is implicated in several congenital anomalies other than NTDs (e.g. craniofacial, limb, heart) but a direct causal relationship is unclear in many cases.

Our research plans:

New genes and environmental causes of mouse NTDs will be identified through hypothesis-led studies and through modelling of candidate mutations from human genomic studies.

Findings from mice will be extrapolated to human NTDs wherever possible. We are members of two international consortia which are carrying out genetic analysis of human NTDs. Gene-gene and gene-environment interactions will be studied by breeding appropriate mutant mice and assessing development of NTDs.

Mouse models with altered folate metabolism will be assessed to allow evaluation of potential effects in multiple organ systems implicated in human disease.

Determining the embryonic mechanisms that lead from genes to birth defects

The focus of mechanistic studies will be on closure of the neural tube, which is the precursor of the central nervous system in the developing embryo. A great deal of information is known about the three phases of neural tube closure: shaping, bending and fusion. Many critical questions remain: e.g. the molecular, cellular and biomechanical basis of each of these events, and how disturbances lead to development of NTDs.

Shaping of the neural plate will be studied in mice mutant for genes we hypothesise are key to this process, such as those in the planar cell polarity pathway.

Bending and fusion of the neural plate will be assessed by analysis of mice mutant for a variety of genetic pathways and by treating embryos with exogenous agents to disturb specific pathways. Use of advanced cellular imaging is a priority.

The adhesion and fusion of the neural fold tips is the final step in formation of the neural tube and will be analysed using genetic mutant mice and advanced imaging.

Developing new methods for prevention of birth defects

Folic acid supplementation is the only method for primary prevention of NTDs available in clinical practice. Two key questions remain: (i) How does folic acid work in the embryo? (ii) Can treatments be developed to prevent those NTD cases that are ‘resistant’ to folic acid?

Our research plans:

We are addressing the mode of action of folic acid and related molecules through studies of mouse mutant strains that develop NTDs and either respond to folic acid, or are resistant.

Our previous studies of the curly tail (Grhl3 gene) mouse mutant showed that while folic acid is ineffective at preventing the spina bifida, inositol is highly preventive when administered either to the pregnant dam or directly to the embryo. We will further examine the mode of action of inositol and other micronutrients in order to identify the molecular mechanism of prevention in the developing embryo. This work will inform our concurrent human studies, in which we have conducted a pilot clinical trial of inositol to prevent folic acid-resistant NTDs and are now starting a large-scale further trial.

We are increasingly testing other possible methods for prevention of NTDs including the use of nucleotides as an alternative to folic acid, and the possibility that formate may be able to prevent NTDs, including hydrocephalus, that result from FOCM defects.

We also aim to develop therapies which can maximise neurological outcomes for individuals who have spina bifida by administering stem cells, genetic constructs or small molecule medications to them in the womb in order to protect the nerves in their spinal cord.

Developing new methods for treatment of Non-Ketotic Hyperglycinemia

Children affected by NKH (an autosomal recessive inborn error of metabolism) suffer profound developmental delay, neurological signs and premature death. There is currently no effective treatment. We developed mouse models with mutation of the equivalent gene (Gldc), which shows similar metabolic disturbances.

Our research plans:

We are examining the biochemical abnormalities and cellular pathogenic mechanisms in Gldc- deficient embryos and post-natal mice to better inform our knowledge of the human disease.

We will examine potential novel treatments for pre-natal and post-natal aspects of the diseases using small molecule/nutrient supplements and gene therapy. This work has already yielded promising results and may lead directly to clinical translation.

Who or what will benefit from these outputs, and how?

The ultimate goal of our research is to make fundamental discoveries on the causation, mechanisms and prevention and treatment of birth defects. This will: (i) contribute to an advancement of knowledge, through open access publication in the international scientific arena, and (ii) enable translation of scientific advances into improvements in clinical practice. Specific benefits may include:

Increased understanding of human prenatal development. Because we are studying a mammalian system, i.e. mice, there is a high likelihood that the principles emerging from our research will be also applicable to the human situation. Moreover, our research can be directly transferred into studies of human embryonic development through use of the Human Developmental Biology Resource which provides human fetal material for studies of gene expression in relation to congenital disease (www.hdbr.org).

Novel methods for genetic diagnosis and genetic counselling. This clinical application should follow the identification of disease-causing genes in mice, provided the findings are confirmed in human studies. For example, families with a history of NTDs are currently given a recurrence risk, during genetic counselling, that is derived from empirical data: that

is, the population frequency of recurrence. However, if our studies lead to a new genetic test, this could predict much more precisely the risk of recurrence in a further pregnancy for any particular family.

Improved understanding of existing preventive therapies. We are particularly studying folic acid supplementation to prevent NTDs, with the goal of understanding how folic acid normalises brain and spinal cord formation in the embryo. This is an important question as use of folic acid supplements or food fortification is widespread worldwide and now being introduced in the UK. Finding an answer to this question could make it possible in future to target folic acid to specific genetic backgrounds, where there is likely to be a benefit, while seeking alternative treatments for genotypes that are non- responsive to folic acid.

New therapies for prevention of birth defects. Our work arising from mouse studies in which inositol was found to prevent folic acid-resistant NTDs was taken forward to clinical trial. A follow up study is now underway and will be underpinned by mechanistic studies in mouse. This could become a generally prescribed treatment within 5 years if the trial outcome is positive. Our research on additional nutritional interventions could further expand the scope for prevention of NTDs.

New treatments for inborn metabolic disorders. Our work on mouse models for NKH will test novel modes of treatment, such as gene therapy, that could be used clinically in years to come, if proven safe and effective.

How will you look to maximise the outputs of this work?

We will disseminate new knowledge via publication in Open Access journals, on our laboratory website, presentation at national and international conferences and via our ongoing interaction with family and support groups focussed on NTDs and NKH.

We will seek to follow-up potential translational opportunities. For example, our previous identification of a preventive treatment for NTDs in mice has led to clinical trial in the UK and a current international clinical trial. Current translational work on NKH has progressed to pre-clinical studies.

We collaborate extensively with other groups to maximise outputs from analysis of individual embryos/mice. For example, in recent studies we collaborated with groups studying skin, ear and palate development, abnormalities of which co-occur with NTDs in some mouse strains.

Species and numbers of animals expected to be used

- Mice: 45,000
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project mainly uses mice, which are used owing to their applicability as a mammalian mode for human development and the conserved effects of genetic mutation. We use mouse strains that carry genetic mutations that predispose to NTDs or other congenital anomalies, or which carry fluorescent reporters that allow imaging or lineage tracing of selected groups of cells. Our work on neural tube closure focuses on early development. Therefore, work is mainly at stages before pain sensation has arisen.

Some genetic mutant mice will be maintained to adulthood (usually as heterozygotes) to be used in breeding and generation of litters for analysis. Pregnant adult mice are culled for collection of embryos. The majority of the work will be under the mild level of severity.

Studies of inborn errors of metabolism requires maintenance of mice to post-natal stages.

Mice that are maintained to post-natal stages will be analysed for experimental outcome, with collection of tissue and blood at the end of the experiment.

Rats are used for production of serum that is used as the medium for culture of mouse embryos at neurulation stages. Adult male rats are used to maximise serum per animal and where possible these are retired breeders that are due to be culled.

Typically, what will be done to an animal used in your project?

Specific details of the experimental approaches and how these relate to Protocols are as follows:

Breeding and genetic typing of mouse strains (including genetically altered)

Breeding colonies of various mouse strains will be maintained and/or obtained from stocks at other designated establishments (Protocol 1). These mice will be either: (i) new or existing models of congenital anomalies that will enable us to extend our analysis of these conditions, and/or (ii) genetically altered mice (e.g. ‘floxed’ gene alleles and Cre recombinase driver lines) that enable us to answer questions about the developmental pathways leading to congenital malformations. Both types of strain are phenotypically normal (with the exception of few strains that exhibit a benign curled tail among heterozygotes), but when intercrossed can yield embryos with phenotypes that will be analysed in the Project.

Strains will be bred (Protocol 1) for four purposes: (1) to maintain the stocks of GA mice; (2) to perform genetic studies to identify the inherited basis of birth defects; (3) to perform studies of gene-gene or gene-environment interactions (by crossing 2 or more different genetic strains) in the origin of birth defects; (4) to provide embryos, fetuses or postnatal offspring for studies on developmental mechanisms of birth defects and other developmental disorders. In many cases, it will be necessary to genetically type the offspring in order to determine their genotype with respect to molecular markers used in the genetic and embryological studies.

Manipulation of embryos in vitro

A variety of embryological analyses will be performed to determine the mechanisms of normal embryonic development and the alterations that result in birth defects. Mouse embryos can grow and develop normally in culture for limited time periods, thereby permitting a variety of research investigations. This is an economical way of conducting experiments since the unit of experimentation is the embryo, not the entire litter. A single

pregnant dam can provide embryos for several different treatment groups, thereby reducing the use of animals overall and reducing the need to perform procedures on conscious animals.

Serum is used as the culture medium for mouse embryos. The serum needs to be prepared in the laboratory by exsanguination via the aorta in preference to the heart, followed by immediate centrifugation of the blood so that clotting occurs in the plasma, without the presence of contaminating red blood cells (Protocol 3). The methods used in preparation of commercially available serum generally do not conform to these requirements, and render the serum toxic to embryos. Hence, we will need to prepare our own serum for culture experiments in this project.

Embryos are typically cultured for a period of 24-48 hours between day 7 and day 11 of gestation. Embryos are not cultured after embryonic day 13.5 (ie, no cultured embryos reach 2/3 of gestation).

Manipulation of embryos in utero

Some studies require that embryos develop for longer periods following a manipulation than is possible with the in vitro system. In addition, for studies of potential therapies it may be necessary to evaluate the effect of maternal administration (e.g. nutrient supplementation/removal). In this case, embryos will be manipulated in utero. Some manipulations of the embryo will be performed on the pregnant female (Protocol 2), by methods including dietary modification, a suitable route such as oral or gavage or via injection or mini-pump implanted subcutaneously using aseptic technique under general anaesthesia. Examples include:

Administration of nutrients such as folic acid or inositol in diet or drinking water.

Conversely, specific nutrients may be withheld by feeding a deficient diet.

Conditional alteration of gene expression may be induced by maternal administration of tamoxifen.

Manipulation of post-natal mice. For mouse models that develop a post-natal onset phenotype, such as the model of NKH (non-ketotic hyperglycinemia) we will administer substances to try to prevent or rescue the phenotype that is found in human patients (metabolic alteration and/or ventriculomegaly) (Protocol 4). Treatments may include dietary modification, oral administration of drugs or injection of substances such as gene therapy vector or drugs (by an appropriate method and volume) or by mini- pump or encapsulated cell pellet implanted subcutaneously using aseptic technique under general anaesthesia.

Neonatal mice (P0-P3) may be administered gene therapy vector by intra-venous and/or intra-cerebral (intra-cerebroventricular) routes in order to achieve transduction of the liver (intra-venous) and brain (intra-cerebral), which are the target organs in NKH. The brain can be readily transduced by AAV at this stage. Mice will then be followed to determine whether the frequency/phenotype of the disorder is altered by the intervention.

In order to identify mice for treatment such as drug administration it may be necessary to genotype pups prior to the stage when they would routinely be marked and genotyped by ear clip. In these circumstances (prior to P7), pups may be marked by toe clip/tail tipped

with the tissue retained for genotyping. This approach will allow avoidance of unnecessary dosing of mice that are not desired genotypes for the study.

Analysis of embryos or post-natal animals after manipulation

A wide range of analytical techniques will be used to measure the effects of the various manipulations in this project. Examples of the types of analysis to be conducted on embryos or tissue following Schedule 1 killing include: Detection of gene expression by in situ hybridisation, immunohistochemistry or histological staining;

Extraction of nucleic acids for transcriptomic or genomic analysis;

Biochemical analysis of homogenates;

Electron microscopy;

Establishment of tissue cultures or organ cultures.

Quantification of metabolites quantified in blood collected under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals produced under the breeding and colony maintenance protocol are not expected to exhibit any harmful phenotype. Some animals may have the potential to develop a harmful phenotype, eg. tumours, neurological signs, after a certain age but in all cases will be killed before reaching that age and before onset of clinical signs, unless moved on to another protocol for a specific purpose (continued use). Animals exhibiting any unexpected harmful phenotypes will be killed (Schedule 1), or in the case of individual animals of particular scientific interest, advice will be sought promptly from the Home Office Inspector. Any animal will be immediately killed by Schedule 1 method if it shows any signs of suffering that are greater than minor and transient or in any way compromises normal behaviour. Advice will be sought from the NACWO and NVS. Humane end points are:

Appearance

Consistent or rapid body weight loss reaching 15% at any time compared with the weight of age- matched mice. In the case of pregnant females, any weight loss over 24 hours. Body weight will also be monitored with reference to Body Condition Score, and culled if BCS is 2 or less – see (Body Condition Scoring; A Rapid and Accurate Method for Assessing Health Status in Mice (Ullman-Cullere & Foltz, Lab. Animal Sci. 49: 319-323, 1999). Under the moderate severity limit, humane end points are listed below and occurence of any three events will be considered the end point:

Piloerect coat showing no improvement over 24 hours
Dehydration and loss of condition showing no improvement over 24 hours

Posture

Hunched appearance showing no improvement over 24 hours

Tremors showing no improvement over 24 hours

Abnormal gait showing no improvement over 24 hours

Paralysis lasting for more than 2 minutes

Repeated episodes of paralysis showing no improvement over 24 hours

Behaviour

Abnormal response to handling impinging on the welfare of the animal (lethargy, extreme hyperactivity)

Unprovoked vocalisation

Persistent head tilt showing no improvement over 24 hours

We will not use mouse strains with overt epilepsy but a few mouse strains experience occasional mild absence-type seizures among homozygous mutant mice. The end point will be occurrence of more than 2 seizures exceeding 30 seconds.

Body Condition Scoring; A Rapid and Accurate Method for Assessing Health Status in Mice (Ullman- Cullere & Foltz, Lab. Animal Sci. 49: 319-323, 1999).

Additional parameters based on Mouse Grimace Scale (https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/MGS%20Manual.pdf) Non-surgical manipulation of embryos in utero Adverse effects on the embryos may be common in the case of administration of certain teratogenic agents. In these cases, it is the purpose of the study to examine the developmental defects that result from treatment of the pregnant female. The embryos will be removed from the pregnant female and killed (by Schedule 1 method if at E13.5 or older) for further analysis so in general, embryos will not die as a result of their developmental defects. In the majority of experiments embryos are analysed prior to two-thirds of the period of pregnancy. Adverse effects on pregnant females are expected to occur in less than 0.1% of cases. Substances that are identified as having such effects will be used only if a non-toxic alternative cannot be found. Prolonged nutrient deficiency can adversely affect the health of mice and non-pregnant animals on restricted diets are therefore monitored twice weekly (as well as daily by animal facility staff) to identify any signs of distress (see below for humane end-points), at which point mice will be killed by a Schedule 1 method. Pregnant females are examined daily for adverse effects, and adversely affected animals will be killed by a Schedule 1 method.

If multiple injections are required the potential complications include infection, pain, local irritation and chemical peritonitis, formation of fibrous tissue and adhesions within the abdominal cavity, perforation of an abdominal organ or haemorrhage. Repeated administration can result in a cumulative irritant effect and needle-induced damage. Before each injection the mouse will be examined for any adverse effects of the previous injection and the multiple injections will not continue if any are seen and the mouse will be culled by a Schedule 1 method.

Adverse effects of surgery to implant mini-pumps: Hypothermia following surgery to implant mini- pumps or subcutaneous cell pellets will be prevented by keeping mice warm post-operatively, and monitoring until full consciousness is regained. Reopening of wounds following implantation occurs in 1-2% of cases. If this occurs, the mouse will be re-anaesthetised and re-sutured on not more than one occasion or, if in distress, killed by a Schedule 1 method. Infection following implantation of mini-pumps is rare (1-2% of cases).

If wounds show signs of infection (e.g. inflamed or weeping wound site) and/or mice adopt a hunched posture and lose body weight and condition, affected mice will be killed by a Schedule 1 method (see below for humane end-points). Non-specific effects of anaesthesia or surgery to implant mini-pumps occur occasionally (see below for humane end-points). Pain will be controlled during surgery by general anaesthesia, and pre- and post-surgery by analgesics (e.g. meloxicam, buprenorphine) as required, with reference to the Horton and Griffith System as recommended by the NVS.

Interruption of pregnancy may be an occasional effect of implantation of mini-pumps. Since mini- pumps are inserted early in pregnancy, the usual result is death and resorption of embryos, rather than abortion. Animals will not be re-mated as resorption is generally only recognised at the stage when embryos are collected. Mice will be monitored for adverse effects twice daily for the first two days post- operatively, then daily thereafter.

When work under terminal anaesthesia is involved, the level of anaesthesia is maintained at sufficient depth for the animal to feel no pain. Humane end points: Any animal exhibiting changes in clinical condition or behaviour as listed below will be killed by a Schedule 1 method, or as advised by the NVS and/or NACWO.

The protocol for production of serum is conducted under terminal anaesthesia. The animal is monitored for level of anaesthesia. A level suitable for surgery has been reached when whisker twitching has ceased and respirations are regular. The animal should not react to pinching of its feet. Great care is taken to ensure that this level of deep anaesthesia is maintained throughout the procedure. On completion of exsanguination, the diaphragm is perforated and death is confirmed by use of a second Schedule 1 method. Blood is centrifuged immediately to separate the serum and then prepared in the lab for use in embryo culture.

Breeding and maintenance of genetically altered animals with potential for post-natal onset phenotype

This may apply to mice with GA of components of folate metabolism, including the Glycine Cleavage System GA mice (loss of function alleles of Gldc or Amt). Heterozygous breeding stock are unaffected. Homozygous GA offspring are expected to show the following clinical signs:

Death at birth: 20% of mutant pups will die at or before birth due to neural tube defects (anencephaly). Most of the surviving mutant pups are overtly normal up to 4 weeks of age.

Approximately, 30% of mutant pups develop gradual-onset hydrocephalus between 4-8 weeks of age.
Mice that are surviving at 12 weeks of age do not develop further clinical signs.

Offspring will be killed at the onset of clinical signs. Hydrocephalus is recognised by a swelling of the cranium followed by gait problems. These mice will be culled immediately the adverse effects are recognised. For rescue experiments the mice will be monitored and any showing adverse phenotypes will be immediately culled and the remaining mice will be monitored for 12 weeks and considered rescued.

Tail tipping may be necessary to genotype animals early (P0-2) to avoid unnecessary dosing in heterozygous NKH mice. This method of genotyping will therefore allow us to reduce by 50% the number of injections in the NKH model. Any animal that shows adverse effects (monitored as below) will be killed by a schedule 1 method.

Neonatal injection: Pups will be re-warmed, observed for re-initiation of movement and immediately returned to the mother. We will observe to ensure that the mother is attending the pups. Potential complication is needle-induced damage. If pups display signs of ill-health (as listed below). They will be killed by a Schedule 1 method.

Humane end points: Following treatment, any animal exhibiting changes in clinical condition or behaviour as listed below will be killed by a Schedule 1 method, or as advised by the NVS and/or NACWO.

Treatment of post-natal mice is intended to ameliorate phenotypes but has potential to have adverse effects. Prolonged nutrient deficiency can adversely affect the health of mice and animals on restricted diets are monitored twice weekly to identify any signs of distress (see below), at which point mice will be killed by a Schedule 1 method.

If multiple injections are required the potential complications include infection, pain, local irritation and chemical peritonitis, formation of fibrous tissue and adhesions within the abdominal cavity, perforation of an abdominal organ, haemorrhage. Repeated administration can result in a cumulative irritant effect and needle-induced damage. Before each injection the mouse will be examined for any adverse effects of the previous injection and the multiple injections will not continue if any are seen and the mouse will be culled by a Schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild (90% of mice)

Breeding and maintenance of GA mice

Non-surgical manipulation of embryos in utero (diet, drinking water)

Moderate (9% of mice)

Non-surgical manipulation of embryos in utero (e.g. intra-peritoneal injection)

Breeding and maintenance of genetically altered mice with potential for post-natal onset phenotype (e.g. breeding of mice of which a proportion may develop hydrocephalus)

Sub-cutaneous implantation (e.g. mini-pump)

Non-recovery (100% of rats)

Production of serum (for embryo culture or biochemical analysis)

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This research addresses the complex questions of (1) how embryos develop before birth, and what can go wrong leading to congenital anomalies, (2) the effect of inborn errors of metabolism in brain development and how can this be normalised by therapy. This can only be studied properly in whole developing embryos and post-natal mice as current cell and organoid technology cannot replicate the complex morphogenetic movements or multi-system physiology of the developing embryo.

Our ultimate goal is to understand the mechanisms of human embryo development and disease as a contribution to understanding the causes and possible prevention of congenital anomalies and the development of treatment for metabolic disorders. However, experimental analysis of human embryos during early stages of development is not practical or ethical. Instead, we will use a mammalian model, the mouse, in order to gain information of maximum applicability to humans.

Research into congenital anomalies concerns the mechanisms by which the embryo/fetus develops its specific shape and associated function, and the disturbed mechanisms that underlie abnormal shape and function. For example, brain and spinal cord function can develop in a sustainable way only if the neural tube closes during early embryonic development. Hence, an understanding of normal development, and of disturbed development that leads to birth defects, requires analysis of whole mammalian animal embryos. Alternatives that have been considered, and which will be used where possible, include:
Direct studies of humans: although descriptive analysis is possible, hypothesis-testing through experimental analysis is impossible, on practical and ethical grounds. We will use human embryos for some descriptive studies, thereby minimising animal usage.

Tissue/cell culture and organoids: although this can provide useful information on certain specific molecular or cellular phenomena, it cannot mimic the complexity of functioning organs, organ-organ interaction or the entire body. For example, some key genes in our studies (e.g. Gldc, whose mutation causes neural tube defects and NKH) are expressed in cell-type specific manner and not in cultured cells. We use tissue culture (2D and 3D) for cellular studies, where analysis of whole embryos or post- natal tissues is not essential.

Computer simulations: although they can be valuable in extending theoretical approaches to embryonic development, they cannot tell us about real biological situations. We will use computer simulation particularly in our studies of the biomechanics of development and congenital anomalies, where data from embryos will be analysed and simulated electronically.

Which non-animal alternatives did you consider for use in this project?

We have made use of bacteria, C.elegans and fish to investigate metabolic changes arising from mutation of genes of interest in our studies. In some studies we replace the use of live animals by examining human embryos, using tissue culture cells and developing computer simulations.

Why were they not suitable?

Mouse and human embryos undergo a closely regulated developmental process and by using mice we can benefit from the array of genetic and molecular tools available as well as access embryos for imaging. For example, we use mouse genetic models with defects in the same genes as cause the equivalent conditions in humans.

While bacteria and worms can be useful for biochemical analysis, they do not undergo the complex development that occurs in vertebrate embryos. Organ development in lower vertebrates such as fish often does not occur by the same mechanisms as in mammals. In particular, formation of the neural tube (a key area of our studies) takes place by a different mechanism in mammals and 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?

Methods for minimising numbers of animals in this project include:
In vitro whole embryo culture. Embryos are removed from a pregnant female and allocated to experimental and control groups. Hence, we do not need to allocate different dams to different groups (reducing numbers of mice used), and there is less need to manipulate pregnant females. Moreover, recent research (funded by NC3Rs) in our group has shown that rat serum can be diluted for use in cultures without compromising embryo development. This will reduce the number of rats that need to be bled for serum production in future research.

Careful experimental design. For experiments with qualitative outcome (e.g. pattern of gene expression in embryos of differing genotype), 15-20 embryos (from 3-4 pregnant females) are examined per group to ensure reproducibility. Where an outcome is quantitative (e.g. phenotype frequency), a power calculation determines sample size and professional statistical advice is sought where needed. Experiments are designed with reference to the ARRIVE Guidelines.

Mice: Breeding colonies of genetically altered mouse strains are used to reveal how birth defects arise. Each strain is bred for: (i) genetic studies to localise the key genes, and (ii) to produce embryos for studies of how birth defects develop. In some studies, embryos growing in a test tube are supplied with nutrients to determine whether spina bifida can be prevented. Mice that are studied for post-natal rescue of NKH are typically maintained to 12 weeks of age. Around 8000 mice per year will be used overall for breeding and mating to produce embryos and experimental offspring for our research. Experimental design, analysis and reporting will conform to the NC3Rs' ARRIVE guidelines. Breeding schemes and experimental protocols will be designed to ensure use of minimum numbers of mice necessary to generate data with sufficient numbers for fully powered statistical analysis.

Rats: Embryos can be grown in a test tube, to minimise the number of pregnant mice used. Rats are killed to produce serum for the embryo cultures. We have developed methods to allow 50:50 dilution of rat serum with serum-free media. This allows a reduction in the number of rats used in this research.
Around 300 rats per year are killed to produce serum for the embryo cultures.

Careful planning of each experiment will allow the estimations of numbers of mice used. For example, the number of mice used in a project involving genetic experiments, we need to breed up to 8000 genetically altered mice per year, in order to generate the 2000 we require for experiments (see Figure 1). This is because we use multistep genetic crosses, in which usable genotypes are present on average only in 25% of offspring. For example, to generate tissue-specific knockout:

Step 1. b-actinCre x flox/flox → b-actinCre; +/- (50% of offspring usable)

Step 2. b-actinCre; +/- (from Step 1) x flox/flox → flox/- (50% of offspring usable)

Step 3. flox/- (from Step 2) x Pax3Cre; flox/+ → experimental embryos (tissue-specific)

Hence, in this breeding scheme, only 1 in 4 mice from Steps 1 and 2 are usable to generate embryos for analysis in Step 3. On the other hand, wherever possible, we use otherwise unwanted wild type embryos for control experiments, to avoid setting up specific wild type matings.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Careful study design to justify for experimental and control groups, use of randomisation and blinding as well as determining sample size are used to minimise the number of animals used per experiment. This will be consistent with the aims of this project as well as to ensure that the results are useful and prevents waste. Sample size calculation and justification are chosen using the MRC guidance document.

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 carried using a small number of animals to test the feasibility of an experimental study to minimise the risk of repeating the experiment or an uninformative under-powered study. For control animals (for example untreated wild type or genetically altered animals) where tissues are collected for analysis, multiple pieces of the tissue will be stored to be used for parallel experiments where equivalent controls are needed therefore reducing the number of control animals used for each experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice offer the most incisive approach to the analysis of birth defects mechanisms, because:

Mouse genetics is understood almost as well as in humans, offering the best possible means for genetic analysis in a mammal;

Congenital malformations in genetically-predisposed mice closely resemble those in humans, providing excellent models for analysis and for evaluation of preventive treatments;

Transgenic/gene knockout technologies offer a sophisticated route towards studying the effects of genes in particular tissues, or at specific stages;

This research project makes extensive use of genetically modified mouse strains. In most studies, living mice are either heterozygous for the genetic alteration, or only mildly affected homozygotes, so post-natal mice in general will not suffer from abnormalities. Our analysis of congenital malformations will be confined in the great majority of cases to homozygous embryos, which are killed at a developmental stage before the onset of pain sensation.
In the small number of experiments involving living mice (moderate severity protocols), the focus of the study is to test treatment for rescue of the phenotype (e.g. to correct metabolic defect and lower circulating glycine levels in a mouse model of NKH). Animals will be culled immediately if ill health becomes apparent (see Protocols for humane end-points). Animals are housed in IVCs with tubes or cardboard shelters to enrich their environment. We do not intend to pursue any severe protocols.

Why can’t you use animals that are less sentient?

The majority of experiments will indeed involve embryos at stages prior to 2/3 of gestation. Adult pregnant mice will be heterozygous (unaffected) and will be killed by schedule 1 prior to embryo collection.

Mammals or higher vertebrates of equivalent sentience are required for the study of neural tube closure and developmental anomalies of relevance to human malformations.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where possible we will use oral administration of drugs as opposed to injection (e.g. as developed for tamoxifen).

Anaesthetic will be used when tail tipping is necessary for genotyping of neonatal mice. Any mouse that has undergone a procedure will be monitored at increased by research staff as well as on a daily basis by Biological Services staff.

Each new experiment will be pre-planned and discussed with Biological Services staff following submission of a pre-study request form.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to the PREPARE guidelines (Laboratory Animals 2018, Vol. 52(2) 135–141) when planning each new study.

Experiments will be conducted in a manner to enable comprehensive reporting in accordance with the ARRIVE 2.0 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular discussions will be taken place between researchers and animal technicians to review current and new 3Rs approaches. Latest 3Rs developments will be kept up to date by monthly NC3Rs e- newsletter and attending NC3Rs events and workshops. We will refer to the NC3Rs website https://nc3rs.org.uk/staying-informed-latest-3rs-advances for additional information.

Previous studies (funded by NC3Rs) have allowed reduction in the number of rats required for production of serum. We also developed methodology for oral dosing with tamoxifen that avoids use of injection and we liaised with NC3Rs to broaden knowledge of this approach.

152. Brain mechanisms and interventions which regulate emotional symptoms in psychiatric disorders

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Emotional behaviour, Major depressive disorder, Cognitive impairment, Psychological, Psychopharmacology

Animal types	Life stages
Mice	adult, aged
Rats	neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the brain mechanisms which regulate affective state-induced changes in behaviour and how they are altered in rodent models of psychiatric disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Depression and anxiety are the most prevalent psychiatric disorders affecting modern society and it is currently predicted that more than 20% of people will experience an episode of mental illness in their lifetime. Although both medical and psychological treatments have been developed, little is known about how they interact with brain mechanisms to have their effects. Importantly, the current medical treatments are at best only partially effective and invariably cause side effects. We also currently do not understand why people develop mood disorders or why their prevalence has increased so dramatically with more than 20% the adult population now expected to experience at least

one episode of depression or anxiety. Studies in humans are particularly challenging as it is technically difficult to gain insights into the underlying brain mechanisms which lead to these disorders. Psychiatric disorders are also highly complex influenced by genetics, early life experience and the adult environment and likely arise from a complex interaction between biological and psychological mechanisms. Animal studies carried out in carefully validated animal models can help us understand the neural mechanism which control relevant behaviours under either normal or pathological states and enable us to work out how current treatments alter these. Animal models also play a critical role in early drug development where they are used to help identify new targets for interventions and to establish the potential effectiveness of new medicines. We have developed new, refined methods to study emotional behaviour in rodents which measures similar behavioural changes in animals to those known to contribute to human mood. This has provided us with the opportunity to undertake translational studies which can ultimately help find new targets for treatment.

What outputs do you think you will see at the end of this project?

My research over the past 15 years has led to the development and validation of methods to study affective states in non-human species which are similar to measures made in humans and are also more refined than previous approaches with the main readouts based on reward-motivated behaviour. A major impact from the planned research will be new information about the mechanisms which control emotional behaviour in relation to both normal animals and those which have been treated to model a psychiatric disorder. Our approach enables us to better understand how currently licensed antidepressant drugs affect emotional behaviour, as this remains poorly understood. We anticipate the knowledge gained in the next 5 years will identify potential new targets for treatment and test whether drugs which act on these targets have antidepressant effects. We will also study the relationship between emotional states and cognition so we can better understand how and why mood disorders also affect people’s abilities to concentrate, make decisions and what they learn and remember. Behavioural changes are central to the symptoms of psychiatric disorders and having translational approaches, as we have developed, enables us to model these in non-human species and then combine behaviour with targeted manipulations of the brain to better understand the fundamental biology of emotions and their impacts on behaviour.

This research will generate outputs in the form of scientific publications which will be disseminated to other scientists and doctors through international journals, conference presentations and lectures presented at scientific meetings. These will improve the wider understanding of emotional disorders and will support colleagues working in academia and industry by generating new knowledge which can be used in the development of better treatments.

Through our collaborations with industry, we directly feed into the drug discovery process. Research outputs from this project will support ongoing drug discovery projects with both large and small pharmaceutical companies with active programmes in antidepressant drug development.

Who or what will benefit from these outputs, and how?

In the short-term our main impact will be through knowledge gain. Our working hypothesis is that a psychological phenomenon known as negative affective biases drives the development of mood disorders and is an important target for treatment. Testing this hypothesis, we would anticipate that the 5-year period of this PPL will enable us to better

understand how current antidepressants are acting within the brain and how these impact on this core psychological process to improve mood and also help to explain why the benefits of treatment vary so much between patients. Dependent on the outcomes of this programme, there is the potential for this work to have clinical implications within the subsequent 10-year period particularly as our work has the potential to improve how we use current antidepressant drugs and psychological treatments and impacts will not depend solely on the development of a new drug.

Our collaborative work with our industrial partners will continue under this PPL and we anticipate this will play a key role in the evidence base needed to support the use of psychedelic drugs in psychiatry. As these drugs already have a well-established safety and tolerability profile, translating our outcomes to patient benefit will not require the same timescale as a novel compound and so may even be realised within the 5 years of this PPL.

As some of the planned studies will relate to novel drug targets there will also be longer-term outcomes (10-20 years) where the fundamental biology discovered over the period of this PPL will be taken forward to inform future drug development programmes. There is the potential over these longer time frames to see the outcomes of these studies leading to new medicines and providing the foundation for drug development programmes.

How will you look to maximise the outputs of this work?

Our aim is to always publish all the results from our studies. We utilise pre-registration and support open science using pre-print servers to disseminate completed studies as soon as possible. The methods we have developed and specialist expertise in translational rodent models for psychiatry research also means we have built an excellent network of collaborators in both academia and industry. Importantly, we not only work with other pre-clinical scientists but with clinical colleagues and researchers utilising experimental medicine approaches in healthy volunteers and patients. These networks support our translational approaches and give us routes to disseminating our findings and maximising the impacts for arising outputs.

Our ability to accurately quantify emotional states in non-human species using unbiased and well validated approaches also has implications for studies into animal welfare.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To understand emotion requires a living, conscious animal which can express relevant behaviours which arise from a complex neural network involving multiple brain areas as well as interactions with the environment. This cannot be replicated in an isolated tissue or cell. Psychiatric disorders are linked to early life adversity and stressful life experiences

during development with symptoms usually presenting in early adulthood. To help us model this, we have included some studies where we manipulate the animal from the early post-natal period. Our behavioural tests are then carried out in adolescent or adult animals. Emotional changes are also commonly seen in aged populations and these are likely to involve different underlying biology to those which develop earlier in the lifecourse and so some studies will also work with aged animals.

Our current approaches have been developed in rats and this species has been able to model important aspects of human psychiatric disorders including how environmental and social factors influence their emotional state. Because rats habituate well to human contact, they respond positively to the types of behavioural tests used and generate reliable and reproducible outputs.

We have more recently started to explore if similar behaviours can be quantified in mice and how we can adapt the methods to suit this species so we can capitalise on the genetic tools available for this species and move at least some of our studies to this lower species. Based on our current experience with these species we are anticipating that some of our objectives can be met using mice, but we will also continue to use rats for most of the systemic pharmacology and studies using non-genetic targeted brain manipulations where they yield more reliable data and show less suffering in association with the types of procedures being used.

Typically, what will be done to an animal used in your project?

A typical experiment for animals used in our mild protocol (~50%) involves non-regulated training and testing in reward-based behavioural tests. In combination with these tests animals may be mildly food restricted (not more than 90% free feeding weight) and animals may be treated up to once daily with a test drug or an acute affective state change induced by a social, environmental or drug treatment. The study may continue for up to 12 months with behavioural testing up to once daily and an acute treatment given using a refined method i.e oral dosing in palatable solutions or unrestrained injection, up to 5 times per week. Where we have a drug treatment which cannot be given using a refined method and requires physical restraint, the number of times the treatment can be given is limited to a maximum of 20 administrations, not more than once daily. In some animals up to 6 additional behavioural tests involving exposure to novel environments and tests of reward sensitivity involving individual housing and ingestion of a palatable solution following up to 24hr withholding of food up to twice weekly may be carried out. In some animals (<10%) an additional step involving blood sampling from the tail vein may be carried on up to two occasions or vaginal smears. All animals will be killed at the end of the protocol including by non-schedule 1 methods to enable tissue collection for post-mortem analysis.

The animals used in the moderate protocol will be either animals which have been treated to generate a model of a psychiatric disorder (~30%) or have undergone a surgical intervention with recovery (~20%) to enable direct manipulation or recording from the brain. In a small number of animals (<10%) they will have both induction of a disease model and a single surgical procedure. These animals will then be used in a similar way to those used in the mild protocol experiencing up to once daily treatments with either pharmacological, environmental or social manipulations and up to once daily testing in reward-based behavioural tasks. Animals may also be tested by exposing them to novel environments on up to 6 occasions and a test of reward sensitivity involving up to 24hr food restriction up to twice weekly. In some animals (<10%) an additional step involving blood sampling from the tail vein may be carried on up to two occasions or vaginal smears.

All animals will be killed at the end of the protocol including by non-schedule 1 methods to enable tissue collection for post-mortem analysis.

To induce the disease models we use methods which recapitulate some aspect of a human psychiatric condition with emotional impairments and their associated risk factor. This may include up to 8 weeks of chronic treatment with up to two different interventions e.g. one pharmacological treatment (once daily injection or oral dosing using refined methods) and one environmental manipulation e.g. early life adversity or adolescents stress. The manipulations do not cause any clinical signs and the animals continue to behaviour normally but show specific changes in our specialised tests.

In animals which have had surgical interventions, behavioural testing may be combined with direct infusions of drugs into the brain via previously implanted cannulae (maximum 16 infusions) or intravenous via previously implanted catheters; optical or electrical stimulation of discrete brain regions and/or recording from the brain involving tethering to enable data transfer. The surgical interventions are done during a single general anaesthetic and with post-operative analgesia and management to minimise pain and suffering. Animals recover from the surgery quickly (<6 hours to return to normal eating, drinking and behaviour) and are returned to group housing within 12 hours unless they have received a head mounted device which is not compatible with group housing (<5% of surgeries).

Animals which have undergone a surgical intervention return to behavioural testing after not less than 5 days and only when they are fully healed. Animals may receive additional drug treatments for up to 6 months post-surgery. The animals which undergo surgical interventions do not develop clinical symptoms or show any signs of changes in their wellbeing compared to non-surgical animals following the post-operative recovery period. In a small proportion of animals, we will induce the disease model and then perform a single surgical step so we can investigate specific neural mechanisms which are altered in the disease model relative to control.

For approximately 5% of the animals used in this project, we will need to use aversive training methods. Aversive training methods may include footshocks, airpuff, loud noise or cues which animals will perceive as a threat. For most studies we use an escapable, aversive stimulation and train animal to predict these and so they can learn to avoid the negative outcome. For these studies, the animals may be tested for up to 6 months. Where an inescapable aversive stimulation is needed (maximum 12 stimulations), these will be limited to a maximum of 6 in any test sessions and not more than 2 test sessions at least 24 hours apart. For both types of aversive training methods we may also use acute pharmacological treatments or use these tests in animals which have previously undergone induction of a disease model or a surgical intervention. No animals will experience aversive training methods in combination with both disease model induction and a surgical intervention.

What are the expected impacts and/or adverse effects for the animals during your project?

For the majority of animals used on this PPL there will be no obvious signs of pain or suffering or abnormal behaviours. Although we will manipulate the animals affective state, this does not change the animal outwardly and are only detected in our specialist assays. The majority of our behavioural testing involves reward-based tasks which are highly enriching and with our handling and habituation protocols designed to reduce adverse

effects, our animals remain easy to handle and actively engage with the experimenter for the duration of the study e.g. up to 12 months behavioural testing).

We use mild food restriction to increase motivation to perform the task for a food reward and to maintain animals at a healthy weight matched to the normal growth curve, but they will experience some hunger for periods of up to 12months.

Animals undergoing induction of a disease-relevant phenotype will experience periods of chronic negative affective state. This is not associated with overt signs of distress or suffering but may be experienced by the animal for up to 6 months.

Animals undergoing surgery will experience some pain from the interventions which will be managed with analgesia and post-operative care and is expected to last no longer than 48 hours with animals returning to normal patterns of behaviour and social housing within 24 hours.

For the studies involving pharmacological and/or psychosocial manipulation, animals may experience transient periods of mild distress caused by the treatment. These will last between ~10 minutes and ~2 hours and will not prevent the animal performing the reward-based tasks. Methods for substance administration have been refined by our group and we use voluntary drug administration in palatable solutions for most studies meaning animals do not require any physical restraint. For rats we can also perform injections using minimal restraint and animals do not develop any sensitisation or aversive behaviours following repeated treatments using this method. Where we do need to restrain the animal, we have limited the number of cumulative experiences. The substances administered are not expected to induce any adverse events. Animals may experience substance administration integrated with behavioural testing for up to 12 months. For studies including aversive stimuli, the type of aversive method and the frequency of simulations are titrated to the individual animals to maintain an active avoidance response. This is done by gradually increasing the intensity and starting with the most refined method. When presented with the cue for the aversive stimuli, animals are able to avoid it happening by performing a specific behavioural response and should therefore avoid the majority of stimulations delivered over the session. Any use of inescapable aversive stimuli will adhere to specified upper limits and will use the least intense stimulus needed to obtain the required behavioural response.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild - Mice ~90%, Rats ~60% Moderate - Mice ~10%, Rats ~40%
What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our primary interest is in the control of higher order behaviours which are only expressed in highly developed animal species and therefore cannot be modelled using non-animal methods or in lower animal species.

To achieve the aims of our project we need to be able to undertake direct manipulations of brain function and then quantify the arising behavioural effects.

Which non-animal alternatives did you consider for use in this project?

Computational models of neural networks

Non-regulated model organisms

Humans

Why were they not suitable?

The complexity of the brain and the types of behavioural process we are studying remain poorly understood and therefore computational models cannot deliver the project objectives. Even in the rat brain which has only a fraction of the neurones of the human brain, we are yet to be able to model even relatively simple behaviours and much more empirical data from humans and non-human animals are needed before this approach could be used to achieve the aims of this project. We continue to support this approach and work closely with computational scientists.

Non-regulated model organisms lack the types of emotional and cognitive behaviours which we are studying and do not have the same complexity in their central nervous systems to humans, limiting their use in modelling psychiatric disorders. We are also particularly interested in the integrated role of psychological, environmental and biological factors and these are poorly represented in simpler non- mammalian organisms.

Humans arguably offer the best model for our research but the questions we want to answer require carefully controlled experimental conditions and manipulations which cannot be achieved in humans for either ethical or technical reasons. We are also interested in evaluating novel drug targets and potential new medicines which are not suitable to be tested in humans. Our animal studies are complemented by our own work in healthy human volunteers and patients as well as work from our collaborators who lead programmes involved in both experimental medicine and clinical trials. We also utilise cell or tissue-based methods where possible, and our animal studies are always designed with careful consideration of the available literature and to test carefully developed hypotheses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated based on our current research funding and the specific objectives defined in those research grants. Each grant is developed with an experiment design annex which details all the planned studies against the programme objectives with their associated sample size estimates. These are all peer reviewed as part of the funding review process. We have also included some animals based on our projected research income and projects which are currently in development or under review with funding bodies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have undertaken meta-analyses of the behavioural methods to provide a reliable estimate of effect size and then used this to design our experiments. Where required, we integrate both the behavioural work and ex vivo studies to maximise the data we can obtained from any one animal and enhance the interpretation of the behavioural studies. For relatively simple studies we can utilise the NC3Rs EDA but where we require more complex designs, we work with local experts in experiment design or our industrial partners who have dedicated statistical support within their organisations.

Depending on the specific objective where possible, we utilise within-subject study designs. This reduces the total numbers of animals as each animal can act as its own control and generate multiple data points which reduced variability. Although this does mean animals may be on protocol for longer or receive more interventions, combined with our refinements, these should not lead to a major increase in cumulative suffering but can dramatically reduce animal numbers.

When we are working with disease models or using chronic manipulations, within-subject designs are not appropriate and for these studies we reduce the numbers of animals by limiting the use of sham or placebo controls and utilise historical data wherever possible. For new interventions or models, we may use a larger number of control groups for our initial studies but can then reduce the numbers for the remaining work and also publish our findings to reduce the need for others to replicate this work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Spare tissues from all our animals are made available through the tissue sharing network at our institution.

We collaborate with computational neuroscientists to maximise the outputs from our behavioural data.

The same animal may be used across a number of behavioural tasks so we can look at individual phenotypes and understand how changes in one aspect of cognition may impact on another. This avoids the need to use separate cohorts for each behavioural test and also reduces variability and overall numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

My research group has pioneered the development of refined methods to study psychiatric disorders in non-human species. We have developed and validated non-aversive behavioural tests which can be used in combination with disease models or acute manipulations of affective state to provide reliable and reproducible measures of emotional behaviour. We have also developed refined housing, handling and habituation protocols which reduce the animal's baseline stress response meaning we can detect more clinically relevant changes in affective state in our animals and without using overtly stressful manipulations. Whilst our readouts are non-regulated, we do need to use methods which generate changes in affective state (positive and negative) and for some objectives, be able to combine these with ways to directly manipulate the brain mechanisms we think are involved or look at how they interact with disease models.

Approximately 50% of our objectives can be achieved without needing to induce a disease model. We work with normal animals and focus our studies on acute pharmacological and/or environmental manipulations of affective state. We use pharmacological treatments at doses which are not expected to cause overt changes in behaviour but can cause short term changes in affective state including causing transient increases in anxiety but not overt fear or distress. We can be confident that the animals are not experiencing high levels of emotional distress as they continue to look and behave normally and it is only through our specific behavioural tests that the effects are apparent. We need to repeat the treatments over the course of a week to obtain our main readout but minimise the interval to not less than 48hrs and not more than two acute negative state inductions in one week. The same animals may be tested using this approach for up to 12months which enables us to compare different treatment effects within the same animal and reduce the overall numbers needed to achieve the objectives. By using refined methods for substance administration and only mild/transient affective state manipulations
e.g. 10min restraint, we can also limit the cumulative impact on the individual animal.
Approximately 30% of our objectives require the induction of a depression-like phenotype so we can understand the changes in behaviour which arise under these conditions and can also test the efficacy of pharmacological and/or psychosocial treatments. We have selected models which avoid the need for induction of overt damage to the brain or use of aversive conditioning methods. It is important that we can test how different risk factors for emotional disorders impact on the behaviours and underlying brain mechanisms of interest and so we have included disease models which each represent at least one of these known risks e.g. genetics, early life adversity, chronic pharmacology, chronic environmental manipulations or natural ageing. These models do not cause any overt signs of disease and are similar in terms of the cumulative impact of the treatments as defined by our affective state readout. We know from human epidemiology that patients often experience more than one predisposing risk factor and so in some studies our animal model will be generated using one developmental intervention, early life adversity or adolescent manipulation, and one adult intervention. It is important for our objective to be able to test more than one disease model, we can gain greater insights into where common mechanisms are impacted and hence the potential for a treatment to have efficacy across different subpopulations or patients or, where a treatment may be particularly beneficial for one specific risk factor.

Approximately 20% of our objectives focus on the specific brain mechanisms which regulate emotional behaviour and these require models where we use a surgical intervention to enable us to directly manipulate the brain or to permit recording from discrete brain regions. There is no alternative for these study objectives, but they will be performed under a single surgical step to minimise the number of general anaesthetics the animal will experience. Post-operative analgesia refines the experience of the animals, and they return to normal behaviour and social housing within 24 hours. Animals may receive a local manipulation into the brain and/or implantation of a head mounted device to enable us to directly administer drugs to the brain immediately before behavioural testing without the confounding effects of anaesthesia or, to enable recordings of brain activity to be taken. None of the regions targeted are associated with causing any clinical signs. A small number of animals will require single housing to protect their head mounted devices and these will be provided with access to a play pen with other animals to reduce the impact. Recording of the data generated by these devices or methods to enable us to directly manipulate the brain include the use of tethering for optical stimulation or recording or local infusions of pharmacological agents. To reduce any distress caused by these methods, animals are habituated to the methods before the experimental sessions and tethering methods which do not restrict the animals natural behaviours are used.

Whilst most of our objectives can be achieved using animals responses to reward-based behaviours, these methods do not enable us to study how animals respond to aversive events but these are important in terms of the wider understanding of emotional disorders. We have limited the numbers of models where aversive methods are used to ~5% and also developed tests which use mild aversive methods such as air puff and aversive stimuli that are escapable however, to generate some models e.g. fear learning, animals may experience inescapable footshock. These will be limited in number and duration and no animal will experience footshock of an amplitude greater than required to induce moderate, transient distress and a reliable freezing response (maximum 0.8mA, maximum duration 1 sec, repeated not more than 6 times in one session and maximum of 2 sessions at least 24 hours apart).

To manage the cumulative experience of any one animal, we limit the combination of steps as much as possible and so only a small proportion of animals (<10%) will undergo both induction of a disease model and a single surgical step and for these studies, animals will not be tested for more than 6 months post-model induction. Some animals may undergo aversive training methods and induction of a disease model OR a single surgical step but no animal will be experience induction of a disease model, surgical step and aversive training methods.

Why can’t you use animals that are less sentient?

We need animals to be conscious and able to perform a complex behavioural task. It is important that we can mirror as closely as possible the development stages of mood disorders and so some manipulations may use immature life stages, but our readouts require a mature adult brain. The majority of our studies utilise the rat as we have found that these animals habituate well to human handling thus reducing non-specific effects of stress, show rapid and reliable performance in the behavioural tasks and are compatible with our refined methods for substance administration which is required for most of our planned studies. We cannot use a non-mammalian species and mice perform the current tasks less reliably and are more susceptible to stress from the amount of human handling required for the behavioural assays.

Some of our planned objectives require the use of genetically modified animals and for most studies these cannot be carried out in rats and so we use mice but with simplified behavioural tasks more compatible with the species but able to deliver the specific objective of the experiment. The use of mice is limited to these specific objectives, and they are not suitable for the majority of the study objectives. As part of our plans within this project, further tasks develops are planned including home cage tests and automation of our current foraging-based tasks and these will be piloted in mice and may be more compatible with this species as they will require less direct human contact. Dependent on progress, we may be able to use mice for more of the planned objectives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals undergo a minimum of one week's habituation to handling (including tickling in rats) which is combined with rewards to enhance the animal's positive association with human interactions. Non- aversive handling methods are used and animals are housed in enriched cages unless the specific scientific objective requires changes to the home cage environment e.g. for a depression model.

Substance administration will use refined methods with animals trained to take drugs from a syringe in a palatable solution or using modified restraint method which we have shown reduces stress responses by ~50%. Some of the drugs we need to test cannot be administered by these refined methods due to their pharmacokinetics and in these incidences, we will use the most refined within the pharmacokinetic constraints of the compound and also limit the number of treatments and associated use of physical restraint.

Animals undergoing surgical interventions will be given appropriate pain management to reduce the impacts of the surgery and recovery will be carefully managed with continuous monitoring until the animal is fully conscious and at least twice daily checks until the animal is fully recovered and returned to normal housing.

For rats which require individual housing due to head mounted devices or animals which are not being regularly tested in behavioural tasks we will use a play pen for further enrichment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have been leaders in the field of refinements for cognitive and affective neuroscience and utilise our objective methods to quantify emotional state in rodents and disseminate our findings through publications and presentations. Our planned research programme has reduced the use of aversive training methods with most of the scientific objectives being achieved using reward-based behavioural tasks.

I attend regular CPD events and assess and implement refinements which are compatible with our research objective. I attend meetings such as LASA and subject specific conferences. I utilise the NC3Rs adn LASA resources to follow the latest guidance on procedures including surgical approaches, analgesia and methods to recognise and manage suffering and also follow developments in the field of laboratory animal welfare in other countries particularly in Europe.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I attend the annual 3Rs symposium held across the local University network and regularly attend webinars and workshops hosted by the NC3Rs. I attend and contribute to national and international conferences in my field enabling me to keep up with the latest developments and also disseminate outcomes and refinements from our own research programmes.

I am currently leading a programme of work on refinements to the housing, husbandry and habituation of laboratory mice and rats and the advances we make in this area will be incorporated into our psychiatry research and associated models.

153. Regulatory mechanisms of acute myeloid leukemia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

acute myeloid leukemia, cancer, therapy-resistance, stem cells, drug targets

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This study aims to identify novel ways that blood cells become cancerous and lead to leukemia development. We also aim to understand the characteristics that render many types of leukemia cells resistant to currently approved forms of treatment. We will determine weaknesses within leukemia cells that can be targeted with new treatments. We will identify new treatments for leukemia patients and undergo pre-clinical testing of novel therapies within our mouse models of human leukemia.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Acute myeloid leukemia (AML) is a highly lethal cancer of the blood and bone marrow that has a five- year overall survival rate of approximately 30%. While patients with specific subtypes of AML respond well to current treatments, many patients fail to enter remission following therapy, and these subtypes of AML are classified as therapy-resistant. Whether

an AML patient responds to treatment is highly dependent on the genetic mutations that drive their disease.

All blood cells originate from a population of cells called stem cells. In AML, genetic mutations occur within the stem cell population that result in uncontrolled cellular division of this population and the inability to properly make all of the normal blood cells needed to be healthy. Current research shows that mutations incurred within specific genes of stem cells are strongly associated with therapy- resistance and poor disease outcomes, but the ways that these mutations promote leukemia development and therapy-resistance remain incompletely understood. Using mouse models of human AML, we can identify how cells initiate leukemia and also identify the weaknesses within these leukemia cells that can be targeted with new treatments. The central aim of this project is to understand how genetic mutations found in AML patients lead to initiation of disease, maintenance of disease, and therapy-resistant disease with the aim of identifying novel therapeutic targets to improve AML patient prognoses.

What outputs do you think you will see at the end of this project?

We will develop highly relevant mouse models for the study of therapy-resistant AML, closely modeling human disease. Using these mouse models, we will investigate the role of genetic mutations found in AML patients in driving disease and in driving chemo-resistance. We will use these models to identify novel therapeutic targets for the treatment of therapy-resistant AML. The findings from this project will be presented in multiple publications in top-tier peer-reviewed academic journals, which our research group has a strong track record of doing. These publications will promote the prompt dissemination of new information such that the AML field can learn from our findings and continue to advance the field of AML research with the aim of improving therapies and patient outcomes.

The specific outcomes expected from this PPL include:
Identify specific biological changes that occur prior to the onset of AML to allow for disease initiation in therapy-resistant AML.

Identifying how specific genetic mutations found in AML patients drive disease initiation, maintenance, and therapy-resistance

Establish new mouse models of therapy-resistant AML that accurately model human AML.

Identify novel therapeutic targets for therapy-resistant AML.

Who or what will benefit from these outputs, and how?

The central aim of this project is to understand the mechanisms by which different genetic mutations found in human patient AML promote disease initiation, maintenance, and therapy-resistance such that we can improve therapeutics and prognoses for AML patients.

Basic science: The work proposed in this project will answer many outstanding basic biological questions in the AML field regarding the specific biological changes that occur prior to the onset of AML and how different genetic mutations function to maintain AML disease progression. These data will benefit the AML research community and the chromatin biology community. Furthermore, to address these questions, we are implementing state of the art single-cell genomics technologies using primary mouse leukemia cells that will benefit the basic biology community as a whole.

Translational science and patients: In this project, we will identify potential novel therapeutic targets using our AML mouse models. Results from this project can significantly improve the poor prognosis for patients suffering from therapy-resistant AML as our findings will directly inform the implementation and pre-clinical testing of novel treatments for AML using the information from these screens. Furthermore, we will generate novel therapy-resistant AML mouse models that will be shared as a resource for the AML research community.

Research community: This work will be disseminated through multiple avenues to ensure that there is not a lag in progress made towards the common goal of improving the survival of AML patients. These avenues include, but are not limited to, presentations at local, national, and international conferences, publication in peer-reviewed journals, and inter- and intra-institution collaboration.

How will you look to maximise the outputs of this work?

We plan to maximize the outputs of this work by:

Optimizing experimental design such that multiple scientific questions can be answered in the same animal. This approach reduces the number of animals needed for experiments while adding no additional stress or harm to the animals being used.

Performing experiments using advanced technologies that require a smaller amount of leukemia cells per experiment. This refinement of more traditional approaches allows us to collect many samples to address multiple scientific questions from the same mouse at the experimental endpoint.

Performing preliminary experiments in cultured mammalian cells prior to performing experiments in mice to optimize the conditions in a system that does not require the use of live animals.

Working through intra- and inter-institutional collaborations to ensure that the data are produced in the most robust manner possible and that the best scientists for the research questions posed are involved in experimental planning and data analysis.

Consulting statisticians for experimental planning and data analysis.

Species and numbers of animals expected to be used

- Mice: 20,000 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

The laboratory mouse is the species of choice for the proposed research for two reasons. First, all of the organ systems that we are studying are conserved between mouse and humans, and the physiology of these organs is similar between the organisms. The physiology and environment of intact organs can only be modelled in an intact animal.

Second, the genome (DNA) of the mouse is easily altered, which allows us to recapitulate identical genetic mutations that are seen in human AML patients and to create those mutations in patterns that are relevant to our overall scientific goals.

Adult mice will be used as their physiology most closely parallels human biology.

Typically, what will be done to an animal used in your project?

We will maintain mouse strains by setting up breedings of males and females. These mice will be minimally handled outside of normal care. Pups will be weaned at 21 days of age.

Experimental mice require a series of injections to turn genetic alterations found in AML patients "on". These injections are administered in the lower abdomen and occur at 8-13 weeks of age. Until these injections occur, the mice do not experience any adverse affects of the genetic alterations as the genetic alterations are not expressed (i.e. "off") until the induction agent has been administered.

For bone marrow transplantation models, experimental mice with the genetic alterations that mimic those found in AML patients will then be euthanized at 10-13 weeks old and their bone marrow cells will be collected after euthanasia. These bone marrow cells will be injected into the tail vein in a recipient mouse that has been irradiated to kill its normal bone marrow cells. Giving a normal mouse bone marrow cells with genetic alterations found in AML patients mimics what is observed in AML patients where they only have genetic alterations in bone marrow cells but not in the cells of the rest of their body.

Blood collections will be taken from the tail by forming a venipuncture. Mice will be returned to their cage when blood clotting at the puncture site has been observed.

Mice will develop AML ranging from 1 month to 18 months following induction of mutations depending on the genetic alterations present. Mice will be euthanized for analysis at the first signs of suffering related to disease onset. For some experiments, animals will be euthanized prior to the onset of disease (a pre-disease time-point). These animals will not experience the adverse side effects of developing cancer.

In some instances, prior to the development of AML, mice will receive treatment with drugs or therapeutics that are administered orally or by injection.

What are the expected impacts and/or adverse effects for the animals during your project?

For breeding: Mice may experience a degree of stress 1) when handled when the breeding is initially established and 2) when litters of pups are weaned from their mothers at 21 days of age. Additionally, there is a small incidence when mothers will harm their own pups. In these instances, the pups usually do not survive. Mothers with a history of this will be replaced by different breeding mothers.
For injections (lower abdomen): Mice will experience a degree of stress when restrained for the injection. Mice will also experience mild discomfort during the injection that will alleviate shortly after the injection has been performed.

For injections (tail vein): Mice will experience an elevated degree of stress when restrained for tail vein injections compared to injections performed in the abdomen. Mice will also experience mild discomfort during the injection that will alleviate shortly after the injection has been performed. As cells injected into the tail vein will eventually lead to the

development of AML in these mice, these mice also have the long-term adverse effect of developing cancer. Mice that develop AML will be euthanized when the first signs of suffering associated with disease are observed.

For irradiation of recipient mice: Mice will experience stress during irradiation due to loud noise associated with the machine, a change in environment, and handling necessary to transfer them to the cage used for irradiation and back to their housing cage following irradiation. Because irradiation kills cells that are important to fight potential disease, mice are at a greater risk for developing infections. They will be fed antibiotic food prior to and after irradiation to combat this.

For blood collection: Mice will experience a degree of stress when restrained for the venipuncture. Mice will also experience mild discomfort during the venipuncture that will alleviate shortly after the laceration has been performed.

For the development of AML: Mice that develop AML will show weight loss (up to 20% of total body weight), pale skin and ears, hunching, lethargy, poor body condition, low blood count, less motility, shaking, and/or difficulty breathing. If any of these adverse symptoms are observed, mice will be humanely euthanized as the experimental endpoint is reached.
For treatment with therapeutics: Mice will experience a degree of stress when restrained for the injection. Mice will also experience mild discomfort during the injection that will alleviate shortly after the injection has been performed. Some treatments may produce adverse side effects in mice including lethargy, weight loss, low blood count, and vomiting.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For breeding: Mild (approximately 95-100% of animals)

For injections (lower abdomen): Mild (approximately 95-100% of animals)

For injections (tail vein): Mild (approximately 95-100% of animals)

For irradiation: Mild levels of stress (approximately 90-95% of animals); moderate due to bone marrow failure (5-10%)

For blood collection: Mild (approximately 95-100% of animals)

For the development of AML: Moderate (approximately 95-100% of animals)
For treatment with therapeutics: Mild (approximately 70% of animals), moderate (approximately 30% of animals)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because leukemia is a blood cancer, we must ultimately perform the majority of our experiments in an animal whose blood and organ systems resemble that of human leukemia patients. The physiology and environment of intact organs can only be modeled in an intact animal. All of the organ systems that we are studying are conserved between mouse and humans, and the physiology of these organs is similar between the organisms.

The stem cells that generate all cell types of the blood grow very poorly in culture. These cells and the closely related populations of cells are often the cell types capable of initiating AML development. Because these cells grow poorly in culture and often do not replicate in culture more than a few divisions, we require these cells to be in their natural environment (a mouse) to answer biological questions regarding leukemia initiation, maintenance, and therapy-resistance.

Our research questions focus on biological changes that occur prior to AML onset (initiation), biological changes that occur to maintain AML progression, and biological changes that promote therapy-resistance. Biological questions pertaining to initiation and maintenance of AML require the physiology and cellular environments of an intact blood circulation system and bone marrow niche. Some questions regarding therapy-resistance can be performed in cultured cells which we have implemented in this project, but must ultimately also be tested in an animal with an intact circulatory system and bone marrow niche to be directly relevant to human disease therapy- resistance. When possible, experiments are first performed in cultured cells and then validated using mouse models of AML.

Which non-animal alternatives did you consider for use in this project?

In many instances, we can perform experiments using leukemia cells in culture as preliminary experiments to focus our hypotheses prior to using mice. We have implemented these experiments throughout the project to reduce the number of mice needed to answer our scientific questions.

We have extensive experience in performing functional genetic screens in cultured cells. We perform these screens in cultured cells derived from our AML mouse models when possible prior to validating targets in mice. However, cells isolated from different AML models do not always grow successfully in culture and, in those cases where AML cells fail to grow outside of an animal, the functional genetic screens must be performed in the AML mouse models directly. When cells derived from AML models do grow in culture, we will always perform functional genetic screens in culture and then use our AML mouse models to validate only the most promising targets.

Why were they not suitable?

Because leukemia is a blood cancer, we must ultimately perform the majority of our experiments in an animal whose blood and organ systems resemble that of human leukemia patients. Cultured leukemia cells are suitable for use in preliminary experiments to narrow down our hypotheses such that we reduce the number of mice needed to answer our scientific questions. Unfortunately, certain cells containing combinations of genetic mutations that induce AML in mice, do not successfully grow in culture (just as

certain types of normal blood cells, i.e. stem cells, do not grow well in culture). In these instances, we are unable to perform preliminary experiments using cells in culture for those specific AML models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have a large amount of experience planning experiments with AML mouse models. We have used knowledge from our previous experiments and power calculations to determine the projected mouse numbers needed for the proposed experiments. Details can be found below.

Breeding mice for project:

Breeding cage: 1 male and 1-2 females
Separate pups from mother (weaning): 3 weeks after birth Estimate new born mice: 6 pups/month

We will initially make a total of 20 breeding cages to maintain our 11 genetic strains of mice, but additional strains will most likely be added during the duration of this PPL necessitating additional breeding cages. We therefore estimate 10,000 mice will be used for breeding.

Experimental mice for project:

We initially plan to generate approximately 30 different AML mouse models over a period of 5 years, some based on genetic crossing and some based on bone marrow transplantation. However, this number may increase during the duration of this PPL. For each transplantation based AML model, we need donor mice (who provide a source of bone marrow to initiate leukemia development) and recipient mice (mice who will be the experimental mice for the AML mouse models). We will also assess the biological events that lead to the onset of AML using a pre-disease analysis time point in each of these models. To determine whether these biological events lead to the onset of AML using statistical methods, we will need multiple mice per model per experiment. We will conduct multiple different experiments per AML model to assess the different biological changes that can occur to change cell behavior and lead to AML development.

For these experiments we estimate we will need 7,000 mice over a period of 5 years.

We will perform experiments in cultured cells to identify new gene targets for therapy to treat AML to replace the need for mice. However, the potential targets that we identify from these cultured cell experiments must be validated in mice to demonstrate that the data we collect in cultured AML cells is also true in an animal model of AML. We will also assess ways to pharmacologically target these potential gene targets identified in cultured cells

using different combinations of therapy on our AML models to determine more effective ways to treat patients with AML.

For these experiments we estimate we will need 3,000 mice over a period of 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animal usage will be minimized by optimizing experimental design. Animal numbers for each experiment are based on statistical analyses that use existing data to predict the minimum number of animals needed to detect a biological change in an experiment. Where possible, our experiments are designed to test multiple questions in a single animal to reduce the total number of experimental mice used. This includes the implementation of novel single-cell technologies for use in biological assays. For example, we have successfully implemented the ability to profile two biological features (chromatin structure and transcription) from the same single cell rather than profiling chromatin structure and transcription in separate experiments.

Experiments that can be conducted in cultured AML cells that have been derived from AML mouse model leukemias will be used to reduce the number of mice needed - following these experiments, mice are used to validate the data collected in an AML animal model.

Genetic strains of mice that do not have an adverse phenotype when combined in the same animal will be combined when possible to minimize the number of breeding cages necessary to maintain the strains used in this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Power analysis calculations will be used to determine the minimum number of mice needed in an experimental cohort to obtain statistically significant data. Our calculations recommend minimum cohort sizes of 9 mice for each new AML model for survival analyses. Based on data collected for our initial AML models, we will adjust these numbers accordingly.

We will optimize biological assays on disease stage and pre-disease stage mice for use with single-cell technologies or for use with a small number of cells such that multiple experiments can be performed on cells isolated from the same mouse to reduce the total number of mice needed for our experiments.

For genetic strains of mice where the genetic mutations are are not expressed until induced with the administration of an agent, we will keep them as homozygous strains to minimize the number of breeding cages needed. Furthermore, in the case where genetic mutations can be combined in a single mouse without any adverse effects, we will combine multiple strains to reduce the total number of breeding mice needed.

For genetic strains that are not currently in use or infrequently used, we will freeze embryos and sperm and re-derive the strains when needed.

For many experimental cohorts of AML, we use bone marrow transplantation as a method to give donor AML cells to otherwise healthy recipients. In this regard, we can keep fewer breeding cages and ultimately fewer mice harboring the genetic mutations that cause AML. Furthermore, unused donor AML bone marrow cells can be frozen and used as

donor bone marrow at a later date, further reducing the total number of breeding mice needed.

In all AML cohorts, we collect multiple tissues from the same leukemic mouse to maximize the information per mouse. This ultimately reduces the total number of AML mice we generate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using laboratory mice as the species of choice for the proposed experiments during this project as the organ systems that we study are conserved between mice and humans and the physiology of the organs is also very similar. AML is a cancer of the blood, and we therefore must use a model organism with an intact blood and organ system that closely resembles that of human AML patients. Additionally, the mouse genome is easily engineered to have specific genetic mutations that are found in human AML patients. We can easily mutate the mouse genome, or take advantage of the countless mouse strains that are already publicly available that contain genetic mutations of interest, to recapitulate the genetic mutations observed in human AML patients for our studies.

During many of our studies, we will use well-established methods to induce genetic mutations in a small number of mice which will be used as sources of donor bone marrow. These mice will be euthanized and their bone marrow will isolated and injected into otherwise wild type recipient mice (whose normal bone marrow has been depleted using irradiation). These mice will develop leukemia in 1 to 18 months depending on the AML model. During this time, we will monitor mice for signs of leukemia development and euthanize mice when any of the humane endpoints have been reached for further analyses.

Why can’t you use animals that are less sentient?

To study AML, we must use adult mice as this age range most closely resembles the physiological state of human AML patients. In most of our AML models, mice take 1 to 18 months to develop disease which necessitates the use of adult mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimize harm to mice while maintaining the genetic mutations needed for the studies proposed, we will use strains of mice with mutations that are not expressed under normal conditions when possible. These mutations are not induced (or "turned on") until the administration of a drug via injection has been performed. These mutations will only be expressed in experimental mice. Additionally, the mutation will only be present in the bone

marrow of the experimental AML mice and not throughout the entire body. This will prevent unnecessary suffering due to effects of the genetic mutations on parts of the body outside of the bone marrow and blood system.

Mice with genetic mutations that are always expressed with the possibility to cause harm will be kept as heterozygous strains (one normal gene copy and one mutant gene copy). This will minimize the chance that the mice will develop adverse effects. If these genetic mutations are known to cause adverse effects at a specific age, these mice will be euthanized prior to the age range of expected onset of adverse effects.

Prior to injection of AML bone marrow cells into recipient mice, we irradiate the recipient mice to deplete their normal bone marrow. To minimize the chance of infection, we will keep these mice on antibiotic food prior to and two weeks after irradiation. For injection of AML bone marrow cells into recipient mice, we perform a tail vein injection. To refine this technique to minimize harm to mice, we will pre-warm the tails of the mice prior to injection to help increase the visibility of the vein (the vein will rise closer to the surface of the skin improving the success of the injection). Furthermore, in many instances AML bone marrow cells will be mixed with wild type bone marrow cells to increase the success rate of the bone marrow engraftment and minimize the chance of bone marrow failure in recipient mice.

Any drugs that are administered orally will be performed by a skilled animal technician to reduce the chance of mis-dosing. Any animals exhibiting signs of mis-dosing will be euthanized immediately via a Schedule 1 or other approved method on this PPL.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidance provided by the NC3Rs webpage: https://www.nc3rs.org.uk/resource-hubs. Here, there is specific guidance to ensure that researchers manage and implement best practices of Reduction, Replacement, and Refinement. Furthermore, we will review the Animals (Scientific Procedures) Act 1986 to ensure that all UK laws are being properly adhered to.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We plan to follow the guidance provided by the National Center for the Replacement, Refinement and Reduction of Animals in Research as stated on their webpage: https://www.nc3rs.org.uk/staying- informed-latest-3rs-advances. A few examples listed in this guidance that we will follow include subscribing to the quarterly e-newsletter, contacting the local NC3R's Regional Program Manager, and attending NC3Rs events and workshops.

154. Supply of Biospecimens from Rats and Mice

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Blood, Tissue, Drug Discovery, Drug Development

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

T
he Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To provide an efficient and humane supply of blood, body fluids and tissues for use supporting pharmaceutical research and development projects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The provision of reliable high quality blood, body fluids and tissues to support in-vivo (animal) studies which themselves can:

Enable new scientific understanding and identification of novel therapeutic mechanisms.
Facilitate demonstration of crossoveractivity between species.
Aid in selection of appropriate compounds for further evaluation in-vivo. Allow calibration and validation of measuring systems.

These lead to better informed decision making in the drug discovery programme and consequently the use of fewer animals on in-vivo studies.

Supplying blood, tissue or body fluids in-house will also result in improved efficiency of use of animals since we will manage requests from multiple functions and co-ordinate supply of bio specimens to ensure the best use of each animal.

What outputs do you think you will see at the end of this project?

Tissues collected will provide material for use for in-vitro experiments which will support the screening of compounds and understanding of tissue interaction.

A reduction of live animals used due to the ability to utilise in-vitro (Out-side living organism) testing of blood, body fluids and tissues.

Projects supported by this licence will have informed decision making to screen compounds for evidence of potential toxicity.

Publications further down the project lines will occur and there may be publications based upon in-vitro studies utilising tissues collected under the permissions of this licence.

Who or what will benefit from these outputs, and how?

All therapeutic areas will be able to utilise blood, body fluids and tissues from this licence providing invaluable information to projects.

Short term - the ability to use tissue from animals will provide information of possible cell interactions with compounds and aid the optimisation of in-vivo models.

Medium term - enable the more effective use of animals within live project licences by optimising endpoints and analysis.

Long Term - Reduce the numbers of live animals used by optimisation of models and screening of compounds allowing us to reduce the overall harm to animals.

How will you look to maximise the outputs of this work?

All tissue requests will be managed by a centralised team ensuring that any animals are utilised to their full potential.

All scientists will be asked to provide the following prior to any work being approved to commence, this will allow us to ensure 3R's is being considered.

Briefly outline the scientific background to the proposed use of the tissues

What are the specific objectives that you aim to address by using these tissues?

How will the tissues be used to answer this scientific question?
Why can you not achieve this objective without the use of animal tissues?

How will the use of animal tissues be minimised?

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will utilise and Adult Rats and Mice, mice may be genetically modified or humanised which will provide us translatable results to further refine in-vivo experiments. These animals are required to provide tissues for use within in-vitro experiments, enabling us to refine our in-vivo experiments. Genetically altered animals may be required to allow for increased understanding of human disease pathway, drug interaction and assessment of new therapies.

Where Genetically altered rodents are required these may be transferred from project licences authorised to supply such animals to project licences authorised to use them.

Typically, what will be done to an animal used in your project?

Animals will housed socially and given environmental enrichment by way of nesting and tunnels.

Animals will experience the on set of general anaesthesia, this may be via inhalation anaesthesia within a induction chamber or injectable method. Onset will be fast acting and animals will be monitored throughout until termination.

Any procedures will be performed under a terminal anaesthesia therefore the animals will have no pain or distress linked to these.

What are the expected impacts and/or adverse effects for the animals during your project?

Transient pain and distress may occur with the induction of general anaesthesia, once the anaesthesia is maintain no additional adverse effect to the animals are expected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals - Non-recovery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In drug discovery, research programmes rely, in part, on biological materials obtained from human or animal sources to validate and confirm disease; associated pharmacological targets and mechanisms for potential new medicines. Wherever appropriate, biological samples from animals are obtained post mortem (after death); however, for some studies where there is justifying scientific evidence (e.g where lack of oxygen will compromise the usefulness of the tissues, or where a high quality and quantity of blood is required), it is necessary to obtain samples from living animals.

This programme of work supports the replacement of using living animals by enabling the supply of high quality biospecimens which in turn facilitates in vitro investigations where primary cells are needed due to lack of appropriate cells from existing sources or it is not possible to use cell culture techniques.

The provision of high quality blood, body fluids and tissues to support in vitro studies is important to: Advance understanding of biological mechanisms of therapeutic interest.

Demonstrate relevant activity of a receptor in different species.

Aid selection of potential medicines for further evaluation of efficacy in vivo. Investigate potential medicines for evidence of safety problems.
Which non-animal alternatives did you consider for use in this project?

There are a number of promising technologies in development including organoid culture, multicellular organ models and ‘organ on a chip’ approaches which aim to utilise human cells to recreate the physiological functions of organs without using animals.

Why were they not suitable?

These approaches do not offer an alternative to replace blood, blood products, body fluids and tissues in a suitable form for use in in vitro investigations required to support the research and development of new medicines at the present 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?

Based upon previous use on the last licence. It is expected that the use of mice will increase due to the increasing use of Genetically Modified animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals chosen to supply any single request is minimised by using collection techniques likely to succeed, including taking blood under non-recovery anaesthesia to ensure that a large volume, non-clotted sample can be obtained, negating the need to use more animals than required.

Biospecimen requests will be co-ordinated in order to supply a number of biospecimens from one animal (e.g. whole blood, pancreas, femurs and liver) to a number of requesters for their individual purposes. This should result in improved efficiency of use of animals (i.e. reduce the total number used).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

It is often possible to co-ordinate collection of biospecimens after death maximising the value of each animal. This may include “banking” and freezing blood and tissue samples.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats and Mice will be used during this project. Some mice will be genetically altered and others contain human tissues.

All licenced procedures to be performed under the permission of this licence will be under terminal anaesthesia therefore pain and distress will be limited to the induction of anaesthesia.

Why can’t you use animals that are less sentient?

Rats and mice are required for use in this licence due to the absence of suitable replacement of animal tissues for assays. These animals will be terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be handled using non-aversive techniques and socially housed with enriched environments to ensure their needs are met and any stress from non-licenced handling and welfare checks are kept to a minimum. Anaesthetic protocols to be used will be discussed with the Named Veterinary Surgeon to ensure they are most suitable for species and strain, with inhalation anaesthesia protocols being utilised where possible.

All animals requiring procedures to be performed under terminal anaesthesia will be continuously monitored by a competent scientist to ensure surgical depth is maintained thought-out the procedure, this will be performed using toe pinch and monitoring depth of breathing for any abnormalities. Animals will also be maintained for terminal procedures on a warming mat.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home office Guidelines, AAALAC and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This project licence is driven to ensure the 3Rs are implemented by the use of only terminally anaesthetised animals.

Publications will be regularly checked to ensure we are optimising tissues.

Continuous professional development will be undertaken by all personal licence holders and Project licence holder to ensure 3Rs are considered and best practices implemented.

155. The influence of sleep dynamics on synaptic plasticity across the lifespan

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

sleep, neuroplasticity, in vivo, rodents, dendrites

Animal types	Life stages
Mice	adult, juvenile, aged
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This research project addresses why sleep is important and how it helps our brain function. In particular, the project aims to better understand how sleep influences our ability to process and retain information and how age influences this ability. This project focuses on the basic molecular and cellular mechanisms that are involved and how the different sleep stages (rapid-eye-movement [REM]/dream sleep and non-REM [NREM] sleep) may have a different, but complementary function 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?

Sleep is an integral part of human life and makes up 1/3 of our lives. Sleep disorders are a major societal problem and are associated with a wide range of human conditions. In fact, most brain disorders during development and ageing are associated with some form of sleep disturbance (e.g., insomnia, sleep apnoea, sleep fragmentation). Natural ageing is also accompanied with notable changes in sleep pattern, especially in an increase in

fragmented sleep. This has led to the idea that poor sleep may be both a symptom and a cause of the progression of many brain disorders. Thus, knowing what sleep does to our brain is essential to understand what goes wrong in human conditions where sleep is impaired. We are addressing these questions in animals where the physiology can be investigated and new insights into basic mechanisms can be obtained.

Our research focuses on synapses, the site where two brain cells communicate. The brain displays a remarkable potential for change in response to new experiences in life and those changes occur specifically at synapses, a mechanism termed synaptic plasticity. Sleep and the ability of the brain to express synaptic plasticity vary considerably across the lifespan, with both evident in high amounts during early life and at lesser levels as we age. This further suggests an important link between sleep and the ability of our brain to adapt to environmental changes. Our study will thus provide important insights on the nature of this link and will establish new starting points for future investigations at both the fundamental and the clinical level.

What outputs do you think you will see at the end of this project?

The direct benefit of this project is to increase our fundamental knowledge of the relationship that exists between sleep and brain function. More specifically, the data collected in this research will reveal how different brain areas changes communication during different sleep stages and how this impacts the molecular landscape at synapses.

Our research will also highlight how all those changes vary across age, which is essential information to understand the beneficial role of sleep across the lifespan.

The indirect, more long-term, benefits of this project relate to the potential for clinical applications. This research combines two types of physiological measures: electrical brain activity and large-scale molecular screenings. Brain activity is measured using surface (electroencephalography, EEG) or intracranial electrodes. While EEG is a widely used method in humans, intracranial electrodes are also used in some patients with neurological disorders (e.g., epilepsy). Our research will try to identify electrical biomarkers associated with ageing and memory performance. Our molecular screening will be essential in revealing which cellular functions are recruited during sleep to help the brain to adapt to new experiences. This will open new avenues for therapeutic targets and will have notable implications for brain disorders known to be associated with alteration of sleep and synapses (e.g., autism spectrum disorders, Alzheimer's).

Results from this project will therefore benefit fields in neuroscience and be of interest for anyone who studies the fundamental and clinical aspects of cognitive functions and dysfunctions and how they interact with sleep. We therefore expect to publish our findings in high impact factor journals.

Who or what will benefit from these outputs, and how?

This project will benefit our knowledge at both the fundamental and clinical levels.

In the short-term, the first beneficiaries will be neuroscientists, computational scientists, and clinical researchers in various fields related to this project (e.g., sleep, brain plasticity, memory, development and ageing).
Data from this research will increase our knowledge on the electrical and molecular changes in the sleeping brain related to learning. Importantly, these changes will be compared across ages, an aspect that is often minimised. Our project is also unique as it

will broaden our understanding on the relative contribution of different sleep stages in all those processes.

The data generated during the project will also be of benefit to computational researchers. This is because we will deliver new, large-scale, data to help model the link between specific physiological measures (e.g., brain rhythms), learning and sleep. Current computational models of memory formation do not take into account the impact of sleep, a gap in knowledge our project should fill.

In the long-term, and beyond the end of this project, individuals may benefit. This is because data from our research may identify new targets to diagnose and treat certain brain disorders. Our research will provide brain activity (e.g., EEG) and molecular data that can be linked to ageing and specific pathologies.

How will you look to maximise the outputs of this work?

Results and data will be presented at international and national conferences during the lifetime of the PPL to share ideas with the broader scientific community. These include large conferences in neuroscience and sleep (e.g., Society for Neuroscience; World Sleep, British Neuroscience Association Festival of Neuroscience) and more specialised conferences (e.g., Gordon Conferences, RNA meeting), the latter being particularly helpful to establish new collaborations. Those conferences are also key to discuss negative results and alternative approaches. Negative results will be included in publications when relevant.

Species and numbers of animals expected to be used

- Mice: 1032
- Rats: 228

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 beneficial role of sleep on brain function involves many different brain structures and physiological systems that can only interact when kept intact *in vivo*. Furthermore, sleep states and brain activity, similar to those known to be important for brain function in humans, are only seen in mammals and can therefore not be studied in lower organisms or simplified systems (i.e., *in vitro*). In this context, the combination of a wide range of molecular and genetic tools, as well as available data on the relationship between sleep and brain plasticity, make rodents our model of choice. All methods (physiological measures and behavioural paradigms) we propose to use have been developed and validated in rodent models. This will help ensure the success of our proposed experiments and will also allow us to provide useful information for comparison with already published fundamental and clinical data in this area.

We are proposing to use ‘young’, ‘adult’ and ‘old’ animals in our studies because sleep and the ability of synapses to modify vary greatly with age. This will allow us to understand

which physiological mechanisms are preserved or changed at different ages, and how brain cell communication during sleep is altered with ageing.

Typically, what will be done to an animal used in your project?

Some animals will be used for breeding purposes only.

Most animals (not involved in breeding or pilot experiments) will undergo one or two brain surgeries and after a period of recovery, a behavioural assessment.

All animals will undergo one surgery to place wireless devices to measure the electrical activity of brain cells. The procedure is performed under general anaesthesia, typically lasts less than 1.5 hours and animals recover to normal behaviour in 2 to 3 days.

A subset of animals will also have fibreoptic probes implanted in the brain during the same surgery and will not require an additional incision. The fibreoptic probes are used to either assess or manipulate activity in specific groups of brain cells. Addition of these probes extends the duration of surgery to about 2 hours but the full recovery period of the animals remains the same (i.e. 2 to 3 days). Animals that receive the fibreoptic probes need to undergo a separate injection procedure prior to the surgery. The injection involves the delivery of a substance into the brain and is necessary to allow the fibreoptic probe to work. This more minor procedure is performed under anaesthesia, lasts no more than 1 hour and has a period of recovery of 1-2 days. Finally, animals may be subject to behavioural experiments. These experiments last a maximum of 72 hours during which brain activity is monitored/manipulated in combination with different types of behavioural paradigms such as housing in the enriched environment cage (up to 12 hours), learning a simple task and/or short sleep deprivation (< 6 hours).

A two-week rest period will be allowed between each surgical procedures and the start of behavioural manipulations. Thus, animals that undergo all procedures undertake these across a minimum of 5 weeks, starting with the brain injection and finishing after behavioural manipulations.

Less than half of the animals (38%) will go through all the procedures and the rest will either only receive one surgery for wireless device placement before behavioural experiments (43%) or used for breeding (13%) and pilot experiments (6%)

To minimise stress, animals will be habituated to behavioural and recording set-ups for several days prior to the experimental phase. At the end of the behavioural experiment, animal will be humanely killed (according to the ASPA Code of Practice) for tissue collections.

What are the expected impacts and/or adverse effects for the animals during your project?

The interventions involved in our experiments include breeding of genetically modified mice, monitoring of brain activity using electrodes, imaging/manipulating brain activity using fibre-optic light stimulation, and behavioural experimentations. These are considered as follows:

Breeding is not expected to generate any adverse effects as these have not been observed previously.

Surgical interventions for brain injections and electrodes/optical fiber probe implantations are expected to cause temporary (1-2 days) pain and discomfort post-surgery which will be alleviated by analgesia. This may include irritation at the site of surgery or where the implant is positioned (i.e. head or dorsal flank).

Behavioural manipulations include short term (6 hours) sleep deprivation, simple learning tasks and housing in an enriched environment, none of which is expected to cause any adverse effects. Some stress is expected when animals are first introduced to the behavioural and recording environment, and this will be mitigated by a habituation phase (3-day) prior to the experiments. Sleep deprivation (≤ 6 hrs) has been kept to the minimum required to test our hypothesis. Housing in an enriched environment is known to increase the animal's well-being.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity for the mice and rats used in this licence is expected to be mild to moderate. The cumulative severity has been noted as moderate due to the two surgeries and behavioural experiments. This will be experienced by 80% of mice and 95% of rats. The remaining animals will either be breeders (15% of mice) or included in pilot experiments (5% of mice and 5% of rats) which are expected to experience a cumulative severity of mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Sleep is a complex state, involving changes in the entire organism and many physiological parameters (e.g., temperature, hormone levels, and type of brain activity). The interaction of all those factors is necessary to fully express the beneficial role of sleep for brain function. It is therefore necessary to study the mechanisms underlying this role in the intact organism (i.e., *in vivo*).

Which non-animal alternatives did you consider for use in this project?
We have considered using *in vitro* approaches (e.g., brain slices, dissociated neurons) and in silico (e.g., computational models) in our research.

Why were they not suitable?

While questions about sleep function have been in some cases addressed using in vitro or in silico models, those approaches are not appropriate to address the scientific questions in our research that relies on dynamic interactions with environmental stimuli. Simplified models (e.g., in vitro) are also inherently associated with disrupted connections between

brain cells and can therefore not adequately reproduce the complete array of biological interactions and mechanisms investigated in this project. To this date, in vitro approaches using dissociated cells in a petri dish are very limited to study sleep functions and poorly recapitulate brain cells activity found during sleep. The other alternative approaches involving mathematical models are also limited to mimic the complex interaction that exists between the brain and our environment, which is a critical process studied under this project. Thus, there is no feasible alternative that would entirely replace the use of a living animal and would allow the project objectives to be met.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated according to the methods and experimental design for each Objective, the expected magnitude of changes in our measures (based on existing literature and experience from the principal investigator), and the information provided by the commercial suppliers (Jax, MMRRC) on the mouse lines for breeding plans. The number of animals and planning of experiments have been established with advice from our in-house statisticians and with the help of the NC3Rs Experimental Design Assistant tool. Of note, all groups will have an equal number of males and females unless specified otherwise. Although we do not expect to see major sex differences in our measures, some studies suggest that sex, especially hormone fluctuations, may influence the ability of brain connections to change. We thus include gender as a variable across ages when we estimated the numbers of animals in each group.

Pilot experiments with small numbers of animal numbers will inform and refine the methods to obtain physiological and behavioural measures.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the NC3R's Experimental Design Assistant (EDA) to help with power calculations for group size and strategy for blinding of experiments. Given the number of factors to consider for each experiment, the statistical methods for data analysis were discussed with our in-house statistician, as suggested by the EDA.

We implemented efficient experimental designs to apply statistical analysis that will assess simultaneously the contribution of several factors (e.g., “age”, “experience”, “brain state”) on our output measures without increasing the number of animals for each experiment

To reduce the number of experimental groups, we will use repeated measures to allow within animal comparisons for some outputs measures (e.g., brain activity, memory performance). The use of telemetry approaches allows the recordings of several parameters simultaneously (electrical activity, movement, brain temperature) in the same animal, which also contribute to reduction.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Other aspects that contribute to optimising the number of animals include:

the development of a breeding strategy using online information from transgenic mouse lines suppliers (Jax, MMRRC) and in consultation with the NACWOs and NVS. The use of transgenic mouse lines will allow to optimise the specificity of our physiological measures, which contributes to both reduction and refinement. The inclusion of pilot studies to establish a reproducible behavioural assessment of memory performance in mice and rats. Pilot studies will also be used to optimise the protocol for animal sample collection and processing prior to its application to animals undergoing the combined physiological and behavioural measures. Those pilot studies will also provide extra tissues to compare results in animals without wireless implants, which is important to validate our findings.

At the end of the experiments, brain tissue and other organs will be harvested. Extra tissue will be made available to other researchers within and outside the University of Surrey.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Sleep states and electrical brain activity, similar to those known to be important for brain function in humans, are only seen in mammals and can therefore not be studied in lower organisms or simplified systems (i.e., in vitro). In this context, the combination of a wide range of molecular and genetic tools, as well as available data on the relation between sleep and synaptic plasticity, make rodents our model of choice to address the scientific questions in this research

All the methods and behavioural paradigms we propose to use have been specifically developed and validated in the rodent model for more than three decades. Of note, in vivo electrophysiological recordings have been applied to rodents for more than 70 years. Over this time a number of refinements designed to reduce suffering have been included such as improved surgical techniques and recovery procedures, reduction of the size of the implants, and non-tethered recordings.

Finally, our research group, including the principal investigator, have extensive experience in rodent behaviour and in vivo brain activity monitoring (electrophysiology > 15 years; fiber optic > 8 years). The wireless telemetry system for EEG measures has been used in-house for several years and procedures have been refined to minimise pain and distress to the animal over the years following the advice from the NVS such as peri-operative analgesics regimens and improvement in the telemetry device and electrodes placements.

Why can’t you use animals that are less sentient?

We are interested in the role of sleep stages and specific brain waves during sleep that have only be characterised in mammals. The questions addressed by our study can therefore not be answered in lower organisms such as flies or nematodes, which are alternative models used to address some questions related to sleep. We use animals at different life stages because we want to specifically characterise the similarities and differences in the physiology underlying the relationship between sleep and brain function across the lifespan. Results obtained in one life stage do not necessarily translate to others and this distinction is critical to understanding the role of sleep in brain function across ages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal purchased from breeders will be acclimatised to their accommodation for at least 7 days prior to the start of any procedures. All animals (purchased or bred in-house) will be familiarised to handling using refined methods such as tunnel or cupping handling.

Animals will be monitored with health checks and score sheets designed for the protocol and ages. The use of anaesthesia and analgesics will be implemented as advised by the NVS to prevent any potential adverse effects, including stress and pain, to the animals.

Old age can be associated with deterioration of general condition and monitoring regimes and health check will be adapted to include signs of age-related diseases so that these subjects can be humanely euthanized. As aged subjects might be more sensitive to temperature fluctuations during anaesthesia, we will consider special recovery heating chambers and parenteral fluid administration or whatever is more appropriate as advised by the NVS for protocols involving surgeries.

To reduce the stress of animals during behavioural manipulations and brain signal recordings, we will habituate the animals to the behavioural and recording cages and devices at least for 3 days prior to the start of the experiments. From the PI’s experience and reports from other labs, rodents habituate well to novel environments, including recording and behavioural arena so we do not anticipate any signs of stress during the habituation period. It has been reported that mice, especially males, can express some aggressive behaviour when put in cage with additional male companions (e.g., enriched environment cage). Any mice that display stress or aggressive behaviour will be excluded from the experiment. Pilot studies will be implemented to reduce the stress of animals during specific behavioural paradigms.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidelines from the NC3Rs and ARRIVE (norecopa # f251b).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The 3Rs are regularly discussed and best practice shared during the BRF user forum at the University of Surrey, which the principal investigator chairs and organises with help from the BRF manager. The principal investigator is also a AWERB member at Surrey and stays informed about on-line sources of information about the 3Rs through subscription to NC3Rs, RSPCA and Norecopa newsletters.

Throughout this PPL, we will review our results regularly and integrate any new knowledge/experience from other publications and collaborative network.

156. Elucidating immune cross-talks in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

immunology, infection, cancer, vaccines, autoimmunity

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this project is to decipher the mechanisms regulating the balance between tolerance and immunity. In other words, how can we fight and clear pathogens and cancer cells while preserving the integrity of our body?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 is a fundamental project which aims to understand how our immune system works in healthy individual, and how it is dysregulated in the context of cancer and autoimmunity. The ultimate goal is to find new strategies to prime or rebalance the immune system to provide new tools to improve vaccines and immunotherapies.

Indeed, autoimmune disease such as type 1 diabetes have no cure. The pancreas cannot make insulin because the immune system attacks it and destroys the cells that produce insulin. Dysregulation of blood sugar can have dramatic consequences which can lead to coma. As such, a better understanding of the mechanisms regulating autoimmune diseases is crucial.

At the other end of the spectrum, cancer is a disease during which our body is tolerant, which means that our immune system does not eliminate it, the way it would do for an infection. Strategies to reinvigorate the immune system has been successful in some instance to treat cancer, but we need a better understanding of why immune responses are inhibited in cancer to create new therapies.

Finally, our work could have implication in strategies to make vaccines against high-mutating viruses, such as Influenza. Influenza is a respiratory virus which causes the flu. It still results in 28,000 hospitalizations and 7,000 deaths in the UK yearly despite current vaccination scheme. This is of particular importance for younger and elderly people, which are more susceptible to Influenza infection.

The healthcare costs of those diseases are substantial and therefore any advances which could be used to design better treatment and vaccines will have a potential impact on those costs by reducing the number hospitalisations. This can create economic benefits by saving health care costs, to allow healthy ageing but also to boost the UK-based pharmaceutical industry.

What outputs do you think you will see at the end of this project?

The anticipated output of this project are multiple:

- new fundamental understanding of the mechanisms regulating immune responses during infection and vaccination
- new information on immune imbalance in tumours, new mechanisms regulating type I diabetes
- peer-reviewed publications describing our work.

Who or what will benefit from these outputs, and how?

In the short term, the scientific community will likely benefit from our findings. Our work will further our knowledge of immune communication and how it relates to keeping our body protected while efficiently fighting pathogens. Other scientists will be able to use our findings and analyse how it applies to their field of interest. In addition, by unravelling the niches fostering this novel way of communication, our work might provide a new way to think about immune regulation.

In the long term, beyond this project, we hope that our work will help other by providing means to improve therapies such as vaccines and anti-tumour therapies.

Amongst other, inhibiting immune communication during carcinogenesis might provide increased anti-tumour responses and help control tumors.

On the other hand, re-establishing or strengthening negative immune feedbacks based on the findings of this project might help the immune system become tolerant to cells in the pancreas, thereby diminishing the symptoms of type 1 diabetes.

In addition, this work will also advance of the mechanisms underlying cross-protection, which is essential for successful vaccines towards high-mutating viruses such as Influenza.

Overall, we speculate that the work contained in this project will have important clinical impacts, all of which have the potential to significantly improve the health and quality of life of patients across a spectrum of ages and disease types.

Ultimately, the creation of new therapies based on our findings could have a dramatic health and socio- economic impact, as cancer and auto-immune diseases are associated with direct and indirect costs. The economic burden of cancer costs £7.6bn a year in the UK. NHS spending on type 1 diabetes is estimated at £2bn and the overall economic burden evaluated at £3.4bn.

In addition, our work could have impact on vaccine strategies against high mutating viruses such as Influenza. Given the high rate of hospitalisation and death in the UK despite current vaccination scheme, any advances which could be used to design better vaccines will have a potential impact on those costs by reducing the number hospitalisations and the healthcare costs. This can create economic benefits by saving health care costs, to allow healthy ageing but also to boost the UK-based pharmaceutical industry.

How will you look to maximise the outputs of this work?

The outputs of our work will be maximised by collaborating with experts in our field, but also experts in fields complementary to ours. For instance, we often collaborate with clinicians to confirm the relevance of our findings in patients. New knowledge will be communicated as early as possible through platforms such as bioRxiv. We aim to publish in open access journals as much as possible to allow access of our findings to everyone.

Species and numbers of animals expected to be used

- Mice: 34800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Despite the significant differences that exist between mice and humans, decades of basic science using laboratory mice strains have shown that such models not only allow a systematic reductionist approach to identify potential molecular candidates that may be relevant to humans, but also help to reveal general principles that regulate biological systems.The similarity between human and murine immune systems is reflected in homology at a genetic, protein and tissue-organisation level. It is estimated that the percentage of mouse genes without any homolog currently detectable in the human genome is less than 1 %, thus strengthening the validity of using mouse models. Furthermore, since we study the immune system, we cannot use invertebrate animals, as they do not have an immune system. Finally, we will use adult mice in this project, as we are studying the mature immune system.

Typically, what will be done to an animal used in your project?

Typically, control animals or animals that do not express a gene of interest will be transferred with immune cells intravenously, then infected, immunised, or a disease will be induced (cancer or type 1 Diabetes). A biological modifier might be injected by the intravenous or intraperitoneal route and animals will be monitored for a period of time ranging from 1h to 3 months. Mice will then be humanely killed.

Mice with tumours will be monitored closely every other day. We check that their tumour is not too big and does not interfere with their behaviour. We also monitor that they do not suffer by checking the way they move and their social behaviour. Usually, mice will be humanely killed after 1 month.

Mice with type 1 diabetes will be monitored for glucose in their urine. If their glucose is not detected, mice should feel normal. Usually, mice will be humanely killed after 1 month.

Mice that have been infected should feel tired for a week. They may display reduced movements, but they should recover within 10 days.

Mice might be exposed to radiation.

Mice might undergo terminal non-recovery surgery.

Animals will not receive more than 16 injections overall.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that will get infected, suffering cancer or diabetes might experience signs of ill-health which includes reduced movement, lack of grooming, hunched posture, or weight loss.

In case of immunisation, those are not expected to occur but if they do, they should be very mild and only transient, a few hours. They might experience flu like symptoms.

Where we induce infection, the lowest dose of the pathogen possible to elicit an immune response. However, the animal will still experience the transient symptoms of infection, such as reduced movement and reduced food intake for up to 10 days before recovery.

In case of carcinogenesis and diabetes, we do not expect most mice to display signs of ill-health, and if they do for more than 48h, they will be killed.

In case of irradiation, most mice will experience ill-health but it should be transient and last less than 2 weeks.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding protocol: 20% mild , 80% subthreshold Experimental protocols: 90% moderate and 10% mild

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The reason we use animals for this project are multiple:

We are studying the immune response to pathogens and disease. There are so far no perfect surrogate techniques that allows us to fully recapitulate the infection or disease state. We need to induce infection and disease in order to be able to study the immune response elicited in these contexts.

The immune response is complex and relies on crosstalk between immune cells and non-immune cells. This crosstalk is orchestrated through the organisation of specific niches which cannot be fully recreated with artificial systems. As such we have to study immune regulation in those niches.

The immune system is spread and circulate throughout the body. This is an inherent feature of the immune system and this is not possible to recreate with other methods.

Which non-animal alternatives did you consider for use in this project?

We considered different types of alternatives:

We will use in vitro or ex vivo models whenever possible to replace, or at least complement in vivo studies. In particular, we will use micro-fabricated devices made of silicone in order to re- create the 3-dimensional component involved in cell migration. Similarly, for some experiments, we will use in vitro methods for tracking real-time migration such as the collagen matrix system. In some cases, we will also use cell culture systems to replace animal tissue in experiments that characterise the biochemical effects of specific signals on T cell migration, activation and functionality. Overall, In vitro techniques and assays can generate important hypotheses regarding the phenomena under study, and we will use them where possible and adequate.

Harnessing human data: using publicly available sequencing data and tumour biopsies.
Why were they not suitable?
in vitro system: while we can and will use those systems in a number of instances, they often don't recapitulate the crosstalk between immune and non-immune cells, which is what we are studying. They also cannot be used to interrogate immune trafficking throughout the body.

harnessing human data: we extensively rely on human data to generate hypothesis and confirm our findings. However, we cannot manipulate humans and human samples in a way that allows us to investigate specific pathways. In addition, we usually cannot keep

live human biopsies long enough to treat with biological modifiers and assess the effect of this treatment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimated the numbers of animals we will need to complete this project based on multiple parameters:

Based on our experience, we have used those models and are familiar with the extend by which immune modifications affect the disease course and outcomes.

Based on the variability of disease course. As for humans, the course of the disease is different for different animals, and we accounted for this in our calculations.

Based on the number of immune perturbations we have to perform. We have designed a list of priority candidates which are the most likely to affect immune regulation and can be manipulated to ameliorate disease.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Appropriate use of statistics and careful experimental design: We are using statistical software to estimate the number of mice we need to analyse the effect of interfering with a given biological pathway, if the effect exists and whether it is robust. We will also use the NC3Rs Experimental design assistant, which will allow us to minimise the number of animals used without compromising our research.

The large numbers of mice for breeding genetically modified mice reflects the use of these mice as donors of immune cells as well as hosts for immune cell transfer or infection.

We will make every effort to minimise random variation and increase uniformity within and between experiments by using age and sex matched mice.

When possible, we will include intrinsic controls to further reduce the numbers of mice required per experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will maximize the use of harvested tissues and cells. For example, immune cells isolated from secondary lymphoid organs from a genetically modified animal may be used as a source of immune cells for in vivo and in vitro experiments. Cells and tissues may be shared by multiple researchers.

Frozen tissues may be archived to permit multi-factorial analysis without additional in vivo experiments (e.g. for using in histology).

We follow best practice in maintaining our colonies to ensure that only those animals needed for our experiments are produced. We also make use of the colony data available to us from the inhouse animal tracking database.

When performing new assays or when we produce a new batch of infectious agent, we will use pilot experiments to determine intra and inter group variations. We will develop in vitro approaches whenever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models: Because of the existence of genetically modified mice, we can analyse the function of a specific cell or gene in a way that is not possible with other vertebrates, making it a model of choice for this programme. For example, some mice strains express fluorescent proteins in specific cells, which allows us to follow them over time and check their location. Also, some mice do not express a specific gene involved in cell communication or cell movement, allowing us to study the relevance of those phenomenon on carcinogenesis or autoimmune diseases.

Tumour induction: We are engrafting mice with tumour cells. We can do this under the skin to mimic melanoma, in the mammary gland to mimic breast cancer, These models generate an immune response which is ineffective, making them the best models to study dysfunctional immune responses and mimic immune responses to cancer in human. Those models have the advantage of being synchronised and do not lead to metastasis.

We will also inject mice with tumour cells intravenously to mimic cancer metastasis to the lung. Indeed, using this route, most cancer cells are trapped in the lung, where they form tumours. This is a commonly used model of metastasis.
We also use models of mice developing spontaneous mammary tumours for their similarity with clinical picture of local growth followed by lung metastasis. Those tumours are detectable by gentle palpation. Lung metastasis can occur with this model, usually after 8 months. We will use models of mice developing skin cancer resembling melanoma in humans. Those tumours develop after topical application of tumour inducing agent. They are visually detectable and lung metastasis usually occurs after 7-9 months.

Overall, we are using well-established models of tumorigenesis that are standard in the field. Both models are the most refined for different purposes. Tumour engraftment models allow for assessment of immune responses without metastasis. The use of spontaneous tumour model will allow us to study how tumours interfere with and subvert the immune system over time to escape it.

Infections: We have carefully chosen Listeria monocytogenes and Influenza as infectious models. Listeria monocytogenes is a bacteria that causes food poisoning and can be fatal for foetuses. The immune response to Listeria monocytogenes is well characterized, and critically relies on the immune cells we study: T cells. Because of its unique intracellular biology that allows Listeria to gain access to the host cell, the study of these responses has served as a paradigm in this context. Furthermore, Listeria-based vaccine are being developed to treat cancer, which makes this model very relevant to our studies. Whenever possible, we will use a more benign strain, such as ActA, but this is not always possible, as this strain is replication incompetent and as such does not fully recapitulate the associated immune response.

Influenza is a virus that causes the flu, a contagious respiratory illness that can be severe for the elderly. The immune response to Influenza is also well characterised and relies on T cells. Influenza mutates very quickly, and as such, flu vaccines usually protect just for a year. We have different strains of Influenza that harbour specific, known mutations, which allows us to mimic how the immune system responds to mutating viruses. The different strains cause similar adverse effects. Their use is important to understand how to generate flu vaccines that could be effective for a longer period.

Immunisation: We aim to use immunisation methods that are well characterised, well tolerated in mice and relevant to human vaccines. We do not expect more than transient discomfort, and they are as such refined methods of immunisation.

Why can’t you use animals that are less sentient?

We are investigating the balance between immunity and tolerance, and for this we need a mature immune system. Therefore we have to use adult mice.

Since disease models and course of infection are long, sometimes weeks or months, we cannot use terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use genetically modified mouse models in which the genes of interest can be activated or deactivated by compounds to reduce the risk of a genetic modification causing harms. These systems allow manipulation in a more controlled fashion, such restricted in time and/or space.

We will make every effort to minimise the number of procedures per animal. For example, given our extensive experience in immunisation protocols, we will choose precise time points to sample blood, reducing the amount of pain, stress and suffering that the animal is likely to experience.

For immunisation experiments, we will use adjuvants that are effective but cause minimal adverse effects. We will not use adjuvants that can cause ulcerations at the injection site. When possible, we will use the least painful injection method.

The pathogens we will use are in common use and well documented. We will only use sub lethal infection dosage for the primary response. We will increase the dose for the secondary/memory responses, but mice will have developed a strong and efficient memory response and eradicate the pathogen very efficiently. Whenever possible, we will

use less virulent strains. For infected animals, we may provide food on the cage floor if we notice weight loss or reduced movement.

Appropriate aseptic techniques will be used in non-recovery surgical procedures. Animals will be monitored before the surgery to ensure that they are fit for the procedure, during the surgery to confirm that they are deeply anesthetised (sufficiently to feel no pain). Animals will be provided with warmth and fluids during the procedure to ensure that hydration and body temperature are still maintained.

For mice irradiation, we will split of the doses of radiation in 2 separate doses separated by at least 3hours to minimise side effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NC3R website and the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We use the NC3R website to stay informed about advances.

In addition, we are monitoring for advertised meetings, event, workshops and courses that deal with the 3Rs and the new advances.

We have regular discussions with the NC3R's Regional Manager, the Named Information officer and animal technicians to review current approaches.

157. Provision of an outsourced drug discovery platform for the development of therapeutics for diseases with an inflammatory component.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inflammation, Drug discovery, Therapy

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project licence is to provide a service to support the development of novel drugs for diseases with an inflammatory component and that have an unmet clinical need.

As part of this work we will improve and refine animal models of disease to ensure they are fit for purpose and are the most appropriate to answer the scientific question when testing new drugs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite notable progress in the discovery and development of medicines, there is still a significant unmet need in the treatment of inflammatory diseases with 50% of all deaths worldwide attributable to inflammation-related diseases (Furman et al. 2019. Chronic inflammation in the etiology of disease across the life span. Nature Medicine, 25, 1822-1832). Inflammation is when the body produces a response against something that harms it, such as an infection, injury or toxin. In some cases, however, this response itself can have a negative impact on tissues and organs resulting in disease. Both short and long-term inflammation have been found to be key factors underlying the development and progression of disorders such as arthritis, allergic respiratory disease, kidney failure, sepsis and inflammatory bowel disease (IBD).

This project will generate important proof-of-concept data in the development of potential new test agents (drugs) targeting these and other inflammatory disease areas. These studies will generate vital information that cannot be found without the use of animals. All clients that we work with are developing test agents which will hopefully contribute to helping people with various inflammatory diseases.

What outputs do you think you will see at the end of this project?

This project will provide important information that aids the progression of new drugs against inflammatory disease through the drug discovery and development pipeline. The information gathered will enable us to identify the most appropriate treatments to take forward to human clinical trials and enable us to quickly determine which drugs should not be progressed any further.

In addition, this work will increase our knowledge of how new drugs work and will help us to identify changes in the body that occur in response to the drug. We can use this further down the line to monitor responses in humans during clinical trials.

Data from studies under this project may also be used to support patent applications and applications by clients for additional funding. Data produced may also support the design of regulatory studies for clients.

Who or what will benefit from these outputs, and how?

Inflammation is associated with a large number of health conditions, ranging from arthritis and chronic kidney disease to allergy, inflammatory bowel disease, autoimmune disease and sepsis. Current medications, however, can often result in serious short and long-term side effects or may not even be available to certain patient populations. Further work is needed to develop safe and effective medicines for a multitude of inflammatory conditions.

This programme of work is expected to enable us to progress new treatments for inflammatory disorders through the key milestones of drug development. Drugs shown to be effective in this project can be advanced into the clinic for testing in patients where they could significantly improve a patient’s quality of life.

How will you look to maximise the outputs of this work?

All studies are designed such that the outputs from each animal are maximised. Expert knowledge is gathered not only from within the preclinical (animal) team performing the animal studies, but from other teams within our company, or our clients’ companies. This ensures that all relevant work that has been performed in the laboratory is taken into consideration when designing animal studies. The *in vitro* (in the test tube) and bioanalysis

teams at our company are experts at analysing tissue and blood samples collected from animals and they help with details of sample collection and storage to ensure that the samples are collected and stored in the best way possible. They are also experts at working with small samples, particularly small volumes of blood, meaning that they can often analyse lots of different biomarkers (a measurable indicator of a disease state or other physiological state) and test agent levels from each animal. Any tissues resulting from projects that can be utilised by other projects will be made available. Our company has a very comprehensive sample record system which makes it very easy to assess the tissues we have banked and the conditions of how they were collected and stored.

In addition, we will seek expertise from our established networks, to ensure that we make use of any new knowledge or incorporate better methods of performing animal studies. We will also use these networks to provide information and training to others on the models and techniques we use in our research. We will maintain good communication with managers of the animal facilities to ensure that any tissues from animals being killed that are not required for our work can be made available to other researchers if suitable.

Although there are times where we will not be able to share animal model information (for example, where it would put us at a competitive disadvantage), we aim to publish or share our findings wherever possible, such as control data or where notable refinements have been made in a disease model or procedure.

Species and numbers of animals expected to be used

- Mice: 3500
- Rats: 2250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are the most common type of animal used for generating 'models' of inflammatory disease and for the testing of new treatments. These models mimic areas of the disease in order to provide data on which drugs are likely to work well in humans as a treatment. The immune system of both species has been highly studied, meaning that a lot is already known about how their bodies work, and the techniques used to mimic the human diseases are well developed.

Adult rats and mice will be used for the work outlined in this project licence as we want the biology of the animals to be fully developed to better represent the patients we aim to treat.

Typically, what will be done to an animal used in your project?

Most animals will be part of studies that aim to test whether new drugs can prevent the development of or treat an already existing inflammatory disease, whether that be arthritis in the joint, symptoms of allergic asthma or bowel disease. In the majority of experiments inflammatory disease will be induced by either single or repeated administration of an inflammation-inducing agent that results in damage to key organs and cells. In other experiments, where genetically altered animals are used, animals may spontaneously

develop disease as they age or an inflammation-inducing agent will be given to induce disease.

As part of the study, animals will be dosed with drugs over a period of days or months. Dosing will often take place on a daily basis but this may vary depending upon the drug. Drugs will be most commonly given by the intraperitoneal (i.p., into the body cavity), subcutaneous (s.c., under the skin) and oral (by mouth) routes and less frequently by the intravenous (i.v., into a vein) or intranasal (i.n., into the nose) routes. For i.p., s.c. and oral routes, conscious animals will be held securely by a trained researcher and the dose administered. For i.v. dosing, animals will be placed briefly in a specially designed rodent restrainer. The animal may be placed into a specially designed warming cabinet for up to 10 minutes prior to restraint. For i.n. dosing, animals may be briefly anaesthetised to allow the dose to be administered directly into the nostrils.

Blood samples may be collected during some studies to measure levels of the drug or to assess how well the drug is working. Blood samples are usually small in volume and are most often taken from a vein in the tail. Where disease is expected to result in changes in behaviour or movement, animals will undergo tests to assess how well they can coordinate their movements, such as completing tasks by finding a treat, or assessing how much they want to explore a new environment. Sometimes, tests will look at more general movements, such as balance and gait analysis (how the animal walks). Anything administered to induce diseases, such as osteoarthritis, should only cause minor changes in the way the animal moves, only detectable by special tests.

Other procedures such as urine collection may be performed. Urine may be passively collected from lightly held animals or where possible using a water-repellent sand on which animals are placed for a short period of time. Rodents urinate freely and with regularity and samples are subsequently collected.

At the end of experiments, larger volumes of blood may be collected from animals under deep terminal anaesthesia. Here, animals are asleep, unaware of any pain, and do not regain consciousness. The animals will then be humanely killed while still under anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

The most common adverse effects in this project will arise as a result of the generation of the inflammatory disease and this will be dependent on the particular disease model used.

Animals administered bacterial or similar agents to induce significant systemic (involves the whole body) inflammation will likely experience subdued behaviour, mild weight loss and slight diarrhoea. We aim to minimise these effects as much as possible and experiments will typically be kept short (typically 72 hours) when using higher doses of inflammatory agent. Animals are monitored closely for weight loss, activity levels, body condition, posture and pain, and killed promptly if the humane endpoints are reached.

Mice genetically altered to mimic clinical lupus (where the body's immune system attacks its own organs) will exhibit increased levels of protein in the urine as well as urinate more frequently. Other symptoms may include swollen glands and in the case of certain strains, itching and skin lesions.

The induction of renal disease will similarly result in an increased amount of protein in the urine as kidney function declines, but at an early stage this causes no suffering or other clinical signs.

The injection of an arthritis inducing agent into the joint will result in discomfort following injection and may lead to the development of painful joints and reduced mobility. These effects may last up to the end of the study (typically 10 weeks).

Animals in which a respiratory allergy is induced will experience multiple instances of anaesthesia. However, it is expected that all animals will recover uneventfully from the anaesthesia with no lasting harm. Exposure to the allergen may result in transient (short) respiratory discomfort such as nasal rubbing, sneezing or discharge. In some cases, animals may experience an immediate reaction to the allergen, resulting in the brief cessation of breathing. This will, however, occur while under anaesthesia and is expected to resolve quickly. Gentle chest massage is performed to support breathing and normal respiration is restored prior to the recovery of consciousness.

In response to inflammation some animals will lose weight, but weight loss is not expected to exceed 15% of the starting body weight or result in long-term reduced activity. The only exception to this is when studying inflammatory bowel disease (IBD), where animals may lose up to 20% body weight but not show any other signs of suffering. In studies of IBD, animals will be administered an agent i.e., through their drinking water, that causes inflammation to the gut. This results in animals failing to absorb as many nutrients through their food compared to normal, causing weight loss. IBD animals may also experience diarrhoea and some blood in their stools.

The drugs given are not expected to cause lasting harm, although sometimes the animal may lose some body weight while adjusting to them. Blood sampling is not expected to cause any lasting discomfort but animals will experience slight distress due to restraint and transient discomfort from needle insertion.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: Mild 33%

Moderate 67%

Rats: Non-recovery 22%

Mild 20%

Moderate 58%

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 understand the effects of potential drugs in treating disease, the whole "system" must be studied. Animals enable us to mimic the whole biological system, allowing us to study how the immune system interacts with other cells and organs. It is not possible to fully study this in isolated cells and/or organs.

Which non-animal alternatives did you consider for use in this project?

Our company regularly uses a range of *in vitro* (in the test-tube) methods utilising cells to understand how a novel test agent might affect the cellular functioning of those cells. From these experiments we can prioritise test agents and only take forward those that have the desired effect and, therefore, look the most promising for the treatment of inflammatory disorders. However, this does not fully answer the research question and studying the whole animal is still necessary.

Why were they not suitable?

None of the alternatives mentioned can replicate the complete model of the human immune and inflammatory systems which are required to accurately evaluate the impact of a test agent on an inflammatory condition. In addition, cell-based work does not test the effects the body might have on a test agent, for example how it gets into the blood, travels around the body and how it is removed from the body. No alternative is currently available that can replace the need to test potential therapeutics in a live animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have analysed the number of animals used previously on projects and typically how many animals it takes to fulfil each kind of study, using the most up-to-date experimental methods. This was then combined with a prediction of likely demand of future projects over the lifespan of the licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have extensive experience in the design of experiments of the types in this project, which has given us confidence in the number of animals required to ensure that no animals are used unnecessarily, but also that the data generated is robust and reliable. We regularly refer to the PREPARE guidelines (https://norecopa.no/PREPARE) and make use of the NC3Rs Experimental Design Assistant (https://nc3rs.org.uk/3rs-advice-project-

licence-applicants-reduction) to ensure that we are using the correct number of animals for every study.

When designing experiments to look at the effect of novel test agents, where the test agent has not previously been dosed before, we look at published literature or client data to determine the variability observed with similar test agents. We can then use the NC3Rs Experimental Design Assistant, or our own in-house developed tool to help determine the most appropriate group size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

If a new disease marker or an inflammatory agent is to be tested or refined, small initial experiments (pilot studies) may be conducted first to make key adjustments before proceeding with the larger experiments. This ensures that the correct number of animals are used when experiments are performed in full.

Data from pilot studies and previous experience are used to ensure that the numbers used are as low as possible, without compromising the reliability of the data. Within our company, a member of the wider team has generated a tool for performing power calculations and can be consulted as necessary to assist with study design. This is regularly compared and checked against similar peer-reviewed versions and adjusted where necessary.

Wherever possible, our *in vivo (*animal) scientists will be blinded to the treatment status of an animal, therefore reducing bias. This enables more reliable information to be gathered from a smaller number of animals. Those who carry out analysis on samples (e.g. blood or tissues) collected during the study are also blind to the treatment status of the animal where possible.

Baseline data (e.g. bodyweight) are recorded and animals are randomly assigned to treatment groups so there is no difference between the groups at the start of the study.

Good planning ensures that within any series of studies we can control for variability that might be introduced by external factors. To limit this variability we use animals of a similar age/weight range, test batches of test agent in the lab first, use the same source of animals and reagents, keep records of all observations made and standardise as many components of an *in vivo* study as is practicable.

Where possible, we will coordinate with other groups to share tissue including post-mortem tissues to reduce overall animal numbers.
Where genetically altered animals are required, these will usually be provided by our internal breeding projects, which will ensure that animals are bred efficiently using as few animals as possible by communicating need with colony managers. Where animals are obtained from external sources, only the number of animals required for the study will be purchased or imported.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals will be housed in a purpose-built, modern and well-equipped facility, that is free of disease- causing organisms such as bacteria, viruses and parasites. They will have access to food, water and items that enhance their environment, such as tunnels, chew sticks and two-storey levels to climb on. Our company staff and the animal care staff are competent in rodent welfare and will ensure that animal suffering is minimised. We aim to house animals in groups to promote normal behaviour. However, aggressive behaviour can occasionally result in animals being singly housed to prevent injury.

Why can't you use animals that are less sentient?

Adult mice are the lowest species of mammal that allow us to adequately study the complexities of human inflammatory diseases. Studies often span weeks to months to look at how disease develops and impacts on the whole organism and how it may be corrected. Therefore, the use of terminal anaesthesia is not a possibility. It is also important that we are able to monitor the behaviour of the animals in a conscious state. This allows us to monitor for adverse reactions to any new test agents administered and also how the disease itself (particularly arthritis and lupus) affects behaviour and movement.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Each project has a dedicated project manager and a team of highly experienced researchers. This enables us to combine years of knowledge and experience and tailor strategies to refine experimental design as well as the procedures themselves in order to minimise harms to the animals. Open and regular communications with other managers throughout the Establishment alongside unit technicians, Named Animal Care and Welfare Officers (NACWOs) and Named Veterinary Surgeons (NVS) further enables relevant and specific care for our studies and to identify any new and better methods that could be utilised.

Animals will be housed in social groups where possible and provided with an enriched environment in order to minimise stress. Animals will be monitored regularly for signs of adverse effects including unexpected inflammation/infection, body weight, activity, responsiveness, condition of coat and posture. Injection sites will be monitored for signs of redness, swelling and infection. Urine samples will be regularly collected, if appropriate, to assess levels of protein in the urine, a biomarker of kidney function. In studies of inflammatory bowel disease where inflammation might cause abdominal pain and discomfort, pain relief may be given when the animals show signs of it (for example, hunching). Should pain relief be required we will consider adding the pain relief to a palatable substance such as Nutella to allow it to be taken voluntarily. Where substances (inflammatory or test agents) are given in the drinking water, other ways of supportive care that don't compromise the experiment will be found, such as dry treats. Where animals are likely to urinate more often due to disease, we will replace the bedding more frequently. Where animals exhibit subdued behaviour, care will be taken to ensure the enrichment in the cage is sufficient to give areas for the animals to rest in a warm environment. This will

include increasing the amount of nesting material available, providing heat pads and mash and/or hydrogel to the cage (if possible).

During studies animal condition and bodyweight is monitored regularly, with body weight recorded prior to each dose administration as a minimum. A scoring system specific to each protocol, where appropriate, will be used to monitor any effects observed either due to repeat dosing or development of the inflammatory condition. Scoring systems will look at factors such as weight loss, piloerection, food and water intake, lesion size and condition, grimace scale, movement and behaviour. This will help us ensure that the condition of each animal is tracked and acted upon as necessary.

All procedures are performed using the smallest needle possible. The lowest volume of blood needed for experiments is determined prior to the study starting to ensure the smallest amount of blood is taken as possible from animals during blood sampling. Water-repellent sand may be used to collect urine passively from animals without the need for restraint. Where possible, animals are regularly handled prior to studies starting to acclimatise animals and reduce their stress during the dosing of agents. Prior to any behavioural testing animals will be acclimatised to the test in order to become familiar with both the operator and environment and minimise stress during testing.

To minimise distress in arthritic mice we will follow measures that include the provision of soft sawdust litter to reduce any irritation on walking, the use of non-tangling nesting material and long nozzles on drinking bottles if movement is impaired. Nutri and hydrogel will also be provided if needed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use PREPARE guidelines for the planning of studies and follow the latest version (2020) of the ARRIVE guidelines for ultimate reporting of data. LASA (Laboratory Animal Science Association) also has a range of published guidance documents with principles that can be applied to our animal studies which are found at https://www.lasa.co.uk/current_publications/.

Additional documents that we will also refer to when conducting our work include:

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

Festing et al. (2016) The Design of Animal Experiments: Reducing the use of animals in research through better experimental design.

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. (E Lilley and M. Berdoy eds.). http://www.lasa.co.uk/publications/

Smith D et al. (2018) Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Animal 51(1S): 5-57. doi: 10.1177/0023677217744587

E. Lilley et al. (2014). Refinement of Animal models of sepsis and septic shock. 2015. Shock, 43 (4): 304-316

Bauman et al. 1994. Pain and distress in laboratory rodents and lagomorphs. FELASA Working Group on Pain and Distress. Lab Animals 28, 97-112. doi: 10.1258/002367794780745308.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

When designing animal studies we consider the appropriate guidelines, including the guidance from The National Centre for the 3Rs (NC3Rs), Laboratory Animal Science Association (LASA), and the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines. This guidance will influence our study design.

158. Estrogen, obesity and signalling in pulmonary hypertension

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

obesity, sex, estrogens, serotonin, pulmonary arterial hypertension

Animal types	Life stages
Mice	adult, juvenile, aged, embryo, neonate
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine the effects of obesity, the sex hormone estrogen, serotonin and sex on the development of pulmonary arterial hypertension. To discover novel therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Many more women develop pulmonary hypertension although men get it more severely. The sex hormone estrogen, the blood chemical serotonin and other 'bad' chemicals are

associated with this disease of the pulmonary (lung) blood vessels. The cells of these blood vessels tend to grow more quickly and thicken the blood vessels, stopping the blood flowing through them properly. Survival is extremely poor even with existing drugs and new drugs are needed to prolong survival.

What outputs do you think you will see at the end of this project?

Novel clinical trials of new drugs, development of novel therapeutic approaches, publications, presentations at international and national conferences.

Who or what will benefit from these outputs, and how?

Our work will inform patients, clinicians and other scientists of novel potential drugs for PAH. In the short-term scientists and clinicians will benefit from this knowledge and our understanding of how the cells of the lung blood vessels change. In the medium term these studies will encourage the development of proof of concept clinical trials. In the longer term, patients will benefit through novel therapies becoming available if full clinical trials are successful. This group has already contributed to the use of PDE5 inhibitors and endothelin-1 antagonists being used for PAH and two novel drugs in clinical trials at the current time (serotonin and estrogenic inhibitors). Hence this work is worthwhile. We will publish the data so that it can either be used to support clinical trial applications or stop others from investigating a drug further in vivo that would not be effective. It is likely that the research will translate into the clinic within 5-10 years.

Other benefits will be: patents on novel therapies, publications (we have published over 20 high impact papers since the start of our last project licence), at least two products advanced to Phase 1 clinical trials, prevention of approaches which are contraindicated (such as non-selective 5HT antagonists), development of refined surgical procedures (such as intra-tracheal delivery of some new therapies) and influence on public policy (for example our work previously showed that estrogen based contraceptives are contraindicated in women with PAH (Pulm Circ. 2015 Sep;5(3):435-65. doi: 10.1086/682230.).

How will you look to maximise the outputs of this work?

Data (both positive and negative) will be published in relevant journals and presented at national and international meetings. We will collaborate with several national and international clinical and non- clinical collaborators.

Species and numbers of animals expected to be used

- Mice: 5000
- Rats: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The models selected are the gold-standard refined models of PAH with high fidelity outputs using advanced Pressure/Volume loop.

We have selected the models for sound scientific reasons. The chronic hypoxic model: In PAH the blood becomes hypoxic (low in oxygen) as thickening and loss of lung arteries means oxygen is not effectively transferred, from the air we breathe into the airways into the arteries. The addition of an injection of the VEGF antagonist sugen prior to hypoxic exposure refines the model such that vascular occlusive lesions appear similar to those seen in patients. Transgenic mice are studied where the genotype infers or protects against a pulmonary hypertensive phenotype. These allow the study of one gene and its influence on the development of PH. We do not anticipate severe adverse events and would not keep animals should these arise causing suffering. Adult animals will be studied.

There might be instances where we need to singly-house animals for welfare or scientific reasons, such as:-we need to measure individual faecal/urinary output and/or food consumption for a prolonged period of time (i.e., re-grouping would not be feasible)-we have stud males for our breeding program and they fight when kept in social groups. Any animals kept singly-housed will be handled with due care and empathy and will be provided with suitable enrichment. If at any point in time the reasons for keeping animals singly-housed ceases to apply, and if in the opinion of animal care staff and/or NVS, it would be safe to return these animals to a social group (especially possible in the case of females), this will be carried out, always under proper supervision to ensure the regrouping is not detrimental to welfare.

Typically, what will be done to an animal used in your project?

Pulmonary hypertension (PH) will be induced by exposure of adult mice and rats (male and female) to normoxia or hypoxia with or without the VEGF antagonist sugen (up to 6 weeks). Mice with relevant genes either missing or over-expressed will also be studied. The gonads may be removed to assess the effects of sex hormones. The development of PH will be assessed under terminal anaesthesia using haemodynamic measurements. Some animals will be treated with drugs or RNA delivered as appropriate to that drug (e.g. subcutaneous injection, intra-venously, intraperitoneal injection, orally or intra-tracheally). Some animals may undergo a schedule 1 kill after the development of pulmonary hypertension for immuno-histological examination of lungs and heart. 10 animals will be treated per experimental group.

What are the expected impacts and/or adverse effects for the animals during your project?

For haemodynamic measurements, these will be carried out under anaesthesia and the animals will be humanely culled before they wake up. Minimal transient discomfort may occur during the induction of anaesthesia. Ear biopsy is the standard way to examine the genes affected in mice and this may involve only transient pain and mild haemorrhage and will be controlled by local pressure and/or cautery.

For gonadectomy, short term pain from surgery and risk of infection will be controlled by general anaesthesia and analgesia and the use of perioperative antibiotics, as advised by the Named Veterinary Surgeon. Should wound breakdown occur, the advice of the NVS will be sought and wound repair permitted on one occasion, otherwise the animal will be humanly killed by a schedule 1 method.

Oral gavage very rarely causes short term damage to the oesophagus and inhalation or drug administration into the trachea or intraperitoneal injection may induce peritonitis or organ damage. All animals dosed via the tail vein or having bloods removed via the tail vein will be monitored closely for signs of tail damage which occur rarely. After exposure to hypoxia, animals lose body weight within the first few days and demonstrate reduced activity. However, acclimatisation then occurs with animals returning to normal activity and eating behaviour, and they begin to gain weight at a normal rate.

All animals will be closely monitored for any of these adverse effects and animals exhibiting any unexpected harmful phenotypes will be humanely killed by a Schedule 1 method, or advice will be sought from the Home Office.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate (exposure to chronic hypoxia, dosing of drugs, high fat diet and gonadectomy 60%). Mild (breeding of transgenic animals 5-10%). Haemodynamic assessment is a non-recovery procedure.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

PAH is a complex disease which involves changes in pulmonary hemodynamics causing changes in right ventricular function. Hence we need to examine the effects of drugs in the whole animal. In parallel to these studies we can examine isolated lungs, hearts and arteries but these do not allow us to determine the effects that changes in the pulmonary circulation have on heart function and so the whole animal needs to be studied.

Which non-animal alternatives did you consider for use in this project?

We can use primary cells from patients to carry out key experiments. We have considered 3D cultures. There are no pulmonary arteries-on-a-chip technologies available at the moment but we could consider these in the future.

Why were they not suitable?

Whilst cells from patients are useful to confirm some key observations, the disease is rare and hence availability of these cells is very rare and so we can only carry out a few key experiments on them. Pulmonary cells are extremely specialised and so there are no alternative cells to these. The 3D co- cultures have a hypoxic core and they are non-reproduceable. In addition, the blood vessels in the lungs and heart have a dynamic

interaction in our bodies and we can only examine this in a whole animal. The cellular work will, however, inform and justify the direction of our animal 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?

These numbers have been estimated based on numbers calculated for studies already funded by the BHF and MRC. 10% extra has been included to repeat selected experiments or they may be used if other funding applications are successful.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The n values for studies are calculated using a power calculation. Otherwise, we will use the NC3R's experimental design assistant or previous experience (ours, or from the literature) to select sample sizes. If additional animals are required to provide tissue for cell culture or tissue harvest for e.g. RNAseq analysis, these may be added to each group according to power calculations dictated by previous experiments that give statistically significant results. If we are in any doubt we consult our Institute statistician for advice. The animals will be kept under procedure for the minimum required time and drugs administered for the minimum required length of time as suggested by the literature or previous studies. We will always choose the appropriate control which will be strain, age and sex- matched. Where hypoxia is used, control normoxic groups will be studied. We harvest and retain tissue from all experimental animals and this can be reused for pilot experiments to e.g. look at distribution of a new potential target in the lung or heart prior to considering in vivo studies. Lungs can also be preserved for years for future studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will harvest, preserve and save tissue from the animals under procedure to be used for new pilot studies and to share amongst other projects and researchers. Blood sampling over time can be carried out sequentially in the same animal to reduce the number of animals required to obtain sequential data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models selected are the gold-standard refined models of PAH which mimic the clinical situation most closely. The chronic hypoxic model: In PAH the blood becomes hypoxic (low in oxygen) as thickening and loss of lung arteries means oxygen is not effectively transferred, from the air we breathe into the airways into the arteries. The addition of an injection of the VEGF antagonist sugen prior to hypoxic exposure refines the model such that vascular occlusive lesions appear similar to those seen in patients. Transgenic mice are studied where the genotype infers or protects against a pulmonary hypertensive phenotype. These allow the study of one gene and its influence on the development of PAH. None of these models develop disease so severely that the animals suffer pain or death. All invasive haemodynamic measurements are done under anaesthesia such that the animal does not wake up after the measurements. All researchers are highly trained, licenced and experienced and we are supported by trained veterinarians.

Why can’t you use animals that are less sentient?

All invasive procedures are done under terminal anaesthesia such that the animal does not wake up after the measurements. The exception is gonadectomy and this needs to be carried out in adult animals so that we can study adult sex hormonal effects.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

This is a project licence with a moderate severity banding, but nevertheless we will seek to implement refinements wherever possible, e.g., ensuring our breeders are at peak age for mating, that they do not have too many litters, that genotyping methods (if required) are the mildest possible, etc. Appropriate anaesthesia is always implemented. Animals that undergo sequential blood sampling by the tail vein are trained to enter a warming chamber to ensure dilation of blood vessels. For gonadectomy, post-operative pain management is implemented. Wherever possible we will also replace frequent dosing by using slow-release drug eluting sub-cutaneous pellets as an alternative.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs, EDA and breeding information.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Information from the NIO and review the NC3Rs website.

159. Improving therapies for blood cancers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Therapy, Paediatrics, Leukaemia, Central nervous system

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This programme of work aims to identify new drug targets and treatments that may be less toxic and better at killing leukaemia and other blood cancers, especially those that have spread to sites outside the bone marrow, such as the brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Childhood leukaemia is the commonest childhood cancer. Although cure rates are good, treatment is prolonged and toxic. Cancer is the leading cause of death for children aged 1-

14 years and despite improvements in survival, leukaemia still accounts for about a quarter of these deaths. Current leukaemia therapy involves the use of intensive multi-agent chemotherapy lasting 2-3 years. Even if cured, many survivors have significant treatment side-effects resulting in late mortality, reduced academic achievement, poor quality of life and accompanying health and societal impacts. The commonest long-term effects are adverse neurological (brain) outcomes seen in 20-40% of patients with leukaemia. This is because current treatment for childhood leukaemia involves intensive brain- directed chemotherapy to prevent the disease returning at this site. Treatment is delivered by up to 26 spinal taps with direct injection of chemotherapy into the spinal fluid around the brain. This is often done under general anaesthetic. This treatment causes many short term (e.g fits) and long-term side- effects (e.g. problems with learning and memory). It is also unpleasant for patients and is very expensive for the NHS. There is an urgent need to develop more effective, and less-toxic, treatments for childhood leukaemia and related blood cancers.

What outputs do you think you will see at the end of this project?

This project aims to discover new ways to treat blood cancer. These results will be made available to doctors and scientists by publishing in high-quality journals.

Who or what will benefit from these outputs, and how?

The results of this project will benefit patients with blood cancer by developing kinder and more effective treatments

They may also benefit the NHS by reducing the costs of treatment

They may also benefit wider society as more children with leukaemia will be cured enabling them to grow up and become effective contributors to society

How will you look to maximise the outputs of this work?

The results of this work will be discussed at scientific meetings with other experts in the field. Any scientific data from the experiments will be made available to other scientists after they have been published. We will collaborate with other researchers and will share our knowledge and expertise as much as possible.

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In our previous work we have shown that if we give mice acute lymphoblastic leukaemia (the commonest cancer in children) then the leukaemia cells grow and spread around the body in a very similar way to patients. Our previous work using these mice with leukaemia has already discovered some important genes that might be responsible for keeping the

leukaemia cells alive. We have shown that the genes we discovered in mice are also important in patients with leukaemia. This means that mice are a very good "model system" for studying how leukaemia spreads to organs outside the bone marrow and how it survives in these new environments. We use young mice (usually 6-8 weeks old) in these experiments because leukaemia is most commonly seen in children. In some experiments we will use older (up to 12 months old) or younger (newborn) mice to allow us to look at the differences between leukaemia in babies, children and adults. Understanding these differences will allow us to develop more personalised treatments for patients.

Typically, what will be done to an animal used in your project?

The project covers a broad range of techniques, but 80% of the time we will use immunodeficient mice injected with human leukaemia cells (called xenograft experiments) and 20% of the time we will use genetically altered mice that develop leukaemia due to the genetic mutations they carry (called genetically altered (GA) mice).

For xenograft experiments the majority of mice will be injected with human leukaemia cells into a vein in their tail (80%) - this is relatively painless and quick. For more delicate samples (such as those taken directly from patients - 20% of cases) they may be put under anaesthetic to allow injection of the leukaemia cells directly into the bone marrow using a fine needle inserted into the leg bone - they will be given painkillers to relieve any discomfort which usually lasts less than a day. The leukaemia cells grow in the mice and spread around the body. The mice have regular health checks and may undergo blood tests/scans/other imaging to look at how fast the leukaemia is growing. These tests are usually done once a week or less. After 4-6 weeks the mice usually start to appear unwell. At this point we will humanely kill the mice to prevent suffering.

For our GA mice experiments (20%) the mice will develop leukaemia at a certain age or when we switch on the faulty gene. The mice have regular health checks and may undergo blood tests/scans/other imaging to look at how fast the leukaemia is growing. These tests are usually done once a week or less. After 2-6 weeks the mice usually start to appear unwell. At this point we will humanely kill the mice to prevent suffering.

For 50% of the mice we will try out new treatments to see if they can get rid of the leukaemia or slow its growth. Treatments can be given by injection or by mouth or added to drinking water or food and generally will last up to 4 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Leukaemia causes:

Low blood counts (anaemia) -causing reduced activity and tiredness - this lasts 1-2 weeks on average

Increased risks of infection (although infections are rare because we keep the mice in specialised sterile caging to protect them)
Weight loss - this is seen in the last 1-2 weeks of the illness

Small tumour formation can occur throughout the body (e.g in lymph nodes, liver and spleen) in the later stages of the disease.

Treatments may also cause side-effects such as loss of appetite, diarrhoea, weight loss or fur loss.

Injection of leukaemia cells directly into the bone marrow can cause discomfort which usually lasts less than a day and is helped by painkillers.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity (causing no more than momentary discomfort) - 50%

Moderate severity (discomfort lasting no longer than 72 hours on any occasion) - 50%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To develop new drugs we need to understand how leukaemia cells adapt to different environments within the body. We are currently unable to recreate these complex biological environments (comprising multiple different cell types, nutrients and blood supply) in test-tubes or tissue culture flasks. By using mouse models, we can not only identify molecules responsible for leukaemic spread or therapy resistance but also modify the leukaemia cells using genetic approaches and/or test new drugs to see if this can be overcome. These are essential to develop new clinic-ready therapies for patients with leukaemia and other blood cancers.

Which non-animal alternatives did you consider for use in this project?

All preliminary work (such as testing whether drugs kill leukaemia cells and what dose to use) will be done in the lab with cells grown in culture, before moving on to experiments in mice. Since we need to study cells taken directly from different sites around the body we have two alternatives - animal models or patient samples taken from the bone marrow and the spinal fluid.

Why were they not suitable?

Cell culture: Our central aim is to understand how leukaemic cells adapt to different environments within the body. We are currently unable to recreate these complex biological environments (comprising multiple different cell types, supporting matrix, nutrients and blood supply) accurately in test-tubes or tissue culture flasks.

Patient samples: Patient material is often very limited and generally does not survive or grow once outside the body. We cannot test experimental therapies directly in humans and cannot genetically alter leukaemia cells and inject them into 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?

I have used statistical methods to determine that I will require around 8-10 mice per experimental group and typically experiments will compare 3 or 4 groups. Based on our current funded work and planned future work we will require 1200 mice per year to perform our research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have used both expertise from statisticians and also the NC3Rs' Experimental Design Assistant. Blood sampling and non-invasive imaging techniques (for example MRI) will be carried out for studies to obtain as much data from a smaller number of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

I will employ the most efficient breeding strategy at all times. Ongoing monitoring of a given cohort (mouse group) allows experiments to be stopped as soon as there is enough data (information), thus minimising suffering whilst obtaining meaningful and publishable results. For any new treatments we will always treat a small pilot cohort to estimate the effect size before performing the full experiment. When possible control mouse cohorts can be shared across studies, to reduce mouse numbers required for individual studies. We always maximise all the information we get from each mouse. During the experiment blood sampling and non-invasive imaging techniques will be carried out to obtain as much data from a smaller number of animals. At the end of the experiment we will analyse as many tissues as possible per mouse by harvesting brain, spleen, bone marrow, cerebrospinal fluid (CSF) and blood and sharing this tissue between different members of our research group working on different projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a variety of mouse models in this project. The models have been developed to be more susceptible to human leukaemia and therefore we can study them without having to expose the mice to harmful irradiation. Other models have fluorescent markers that allow more straightforward investigation of processes within the organ(s) of interest using imaging without the need for additional invasive procedures. All these models were developed to cause the least pain, suffering or distress possible while providing us with valuable data on the disease of interest.

Why can't you use animals that are less sentient?

Mice are still sufficiently closely related to human beings to adequately represent the human condition, whereas other model organisms more distantly related would not replicate the disease sufficiently (on a genetic and disease characteristics level). Most of these experiments will be done on young mice, as we are studying childhood blood cancer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal monitoring, post-operative care, and pain management are an important part of all the procedures within the project. The need to minimise suffering is always considered when planning experiments. None of our experiments exceed a moderate severity level. All mice on experimental procedures will be frequently monitored (minimum 1 x weekly, increased to daily as needed) and humanely culled when exhibiting signs of altered health status and/or tumour burden or another specified endpoint is reached. We have developed, and successfully used, a stringent distress scoring system that allows an immediate identification of mice with adverse effects. Wherever possible we will use minipumps to deliver drugs to overcome the need for daily injections, therefore reducing the stress of repeated procedures. All researchers working on this project will undergo specific training in monitoring leukaemia development and health status in our models. We will refer to the literature for adverse effects of a new agent and when a genetic cohort is given a treatment for the first time, studies with a pilot-sized group will be carried out and closely monitored before extending to a larger cohort. All animals are housed in a dedicated facility proactive with environmental enrichment and the use of anaesthesia and analgesia under guidance from the named vet is routine practice. To minimise infections immunocompromised mice, will be housed in barrier caging under sterile conditions and handled in a Class 2 cabinet. Post-mortems are carried out to investigate any unexpected deaths.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
For all studies we will refer to the Guidelines for the welfare and use of animals in cancer research (Workman et al, 2010) and ensure best working practice. We consult the NC3Rs guidelines and monitor refinement when practice advances are published (https://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All our work observes government guidelines we adopt new guidance as soon as these are recommended. We are also actively involved in our establishments 3Rs Day, and there are a number of Culture of Care events organized throughout the year to ensure everyone

stays up to date with current guidelines. I will also 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 licence holders working on the project.

160. Provision of Biological Materials and Research 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

Key words

Immunology, Diagnostic, Disease prevention, Vaccine development

Animal types	Life stages
Cattle	juvenile, adult, neonate, pregnant
Sheep	juvenile, adult, pregnant, neonate
Pigs	juvenile, adult, neonate, pregnant
Geese	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This Project Licence aims to provide scientists with biological reagents (blood and blood products) and neonatal animals of a known health status to support the research of the establishment and programs of research at other collaborating establishments and institutes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 immune responses greatly aids in the development of vaccines and other interventions that can help prevent or minimise disease and thus improve animal health, welfare and productivity.
There are animal diseases that are difficult to diagnose and prevent and there are new, emerging diseases of livestock which science needs to understand in order to treat and prevent.

In the investigation of disease and the development of vaccines and other beneficial interventions, occasionally scientists may require small volumes of normal blood for purposes other than those defined in their own Project Licences. Additionally, some completely lab-based scientists may not have a Project Licence as they do not work in the whole animal. This may be allowed under the conditions of this licence provided the cost/benefit analysis for the procedure is deemed to be of sufficient merit by the Animal Welfare and Ethical Review Body (AWERB) and the procedure does not exceed the Home Office guidance on minimal severity protocols.

Surgical delivery of new-borns allows production of Specific Pathogen Free (SPF) animals, where disease risk and variability due to pathogens is eliminated. Scientists whose Project Licences cover the procedures they will perform on animals of a known pathogen exposure usually do not have surgical delivery on their own Project Licence.

What outputs do you think you will see at the end of this project?

This project will provide support to researchers working to promote livestock and one health through increased understanding of animal immune responses. This will aid the development of diagnostic tests, preventative and treatment strategies. Researchers are encouraged to publish study findings with the knowledge gained being shared through collaborations, publications and conferences.

The production of animals by surgical derivation is required to produce animals that have no exposure to pathogens which could cause disease and variation. This allows study of immune responses to a particular challenge, and the pathology resulting from it, without the potentially confounding or confusing effects of intercurrent acquired infections.

Who or what will benefit from these outputs, and how?

This Project Licence allows for the provision of blood products for use by scientists at the Establishment or other organisations (universities, hospitals and research institutes). This will benefit the development of diagnostic tests, preventative and treatment strategies to improve livestock health. Benefits may also be applied to a One health concept and improve human health.

For example, goose blood is utilised for clinical and surveillance purposes to test ruminants and other wild and managed animals (hare, grouse) for Louping ill, for clinical and surveillance purposes. Louping ill (LI) is a viral disease (Louping ill virus, LIV) transmitted by sheep ticks (Ixodes ricinus) and has been recorded for more than 200 years in Britain in sheep flocks. Louping ill virus (LIV) is mainly detected in sheep, cattle, red grouse and ticks in upland areas of the British Isles, particularly in Scotland, Cumbria, Wales, Devon and Ireland. It has also been detected in a range of other animal species, including goats, dogs, pigs, horses, deer, llamas, alpacas and mountain hares and, occasionally, in human. This test is of national importance with the establishment being the only one providing it.

Goose blood is also used as a feed source to maintain poultry red mite colonies. Artificial rearing of colonies aids the development of vaccines against this serious health and welfare concern of the poultry industry.

The blood samples are crucial to the development of novel vectors for vaccine delivery in sheep. Currently, we use these vectors in pre-clinical cell culture studies and the blood samples provide a source of appropriate primary cells for culture. We expect these vaccine delivery vectors to lead to improved future vaccines for controlling infectious diseases in sheep and cattle. Improved disease control will reduce economic losses due to disease and alleviate the animal suffering caused by those diseases. Reduction in disease of farmed animals also reduces the environmental impact of farming.

Whole blood donation may be used in *in vitro* cell culture immunological assays. Sheep and cattle blood is required to isolate and grow the potentially zoonotic bacteria Anaplasma phagocytophilum, the cause of Tick Borne Fever. This is a tick-transmitted disease widespread in northern Europe and the UK causing severe disease in ruminants and other mammals, including humans.

The production of SPF animals by caesarean derivation is required to produce animals with no maternal antibodies for use in studies under a subsequent Project Licence, and on occasions for studies where animals of seronegative status, for specific antibodies, are required to comply with either Pharmacopeia guidelines or specific study requirements.

In some cases SPF animals are required for both safety and efficacy testing of veterinary products. Some of the studies required for the regulatory submission for new products need to be carried out in the most naïve category of animals in which the product may be used in the field. A seronegative animal is generally considered to be the most naïve animal. In some cases it is possible to source antibody negative animals for particular diseases from the field which can then be reared conventionally, which removes the need for caesarian-derived SPF animals. However, for ubiquitous organisms and in the case of endemic disease, it is often not possible to find animals in the field which are seronegative, as all will have been exposed to the organism, in the environment or the dam's colostrum. In such instances, SPF animals are required.

Where possible, colostrum-deprived animals can be substituted in place of caesarian-derived animals and this is done under a different Project Licence.

How will you look to maximise the outputs of this work?

Publications and knowledge exchange is encouraged from the research groups benefitting from this licence. Data is provided to commercial clients to develop new bioactives used in the treatment and prevention of disease.

Serosurveillance, using goose blood to detect LIV, is generally used to identify pastures where possible tick exposure might be likely therefore avoiding exposure not only to LIV but also to other tick borne diseases and to monitor disease prevalence in grouse moors.

Species and numbers of animals expected to be used

- Cattle: 100
- Sheep: 200
- Pigs: 120
- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mature healthy animals are the only suitable source for blood products required. The animals are selected based on cell types and blood properties specific to the disease being investigated.

The production of SPF animals provides a naive animal model that is not affected by passive immunity nor variability due to exposure to a pathogen.

Typically, what will be done to an animal used in your project?

The animals of farmed species are kept in a natural farm environment, indoors or outdoors, in company, and the majority will be used to provide small blood samples at specified, relatively infrequent, intervals. Frequency and volume of blood taken will follow local welfare guidelines. Animals will restrained by normal farm practice and blood withdrawn by experienced, trained PIL holders. Other than mild discomfort associated with needle-prick there should be no adverse effects and the Severity is Mild. The procedure is quick and animals are returned to their group.

Animals kept alive at the end of procedures will be returned to stock, under the care of a veterinary surgeon with knowledge of the lifetime experience of the animal, who has determined that the animal has been fully restored to health.

Full term dams (sheep, pigs) will undergo general anaesthesia to allow surgical delivery of offspring. The dam will not regain consciousness and will be euthansed.

What are the expected impacts and/or adverse effects for the animals during your project?

All blood sampling procedures on the licence are of mild severity and no adverse effects, other than mild discomfort from the needle-prick are expected. This will be of a brief time duration in the majority of animals.

Each subsequent blood sample is a reuse and then animals are returned to stock (kept alive at the establishment). This information is kept on a database. Some animals are humanely euthanased for reasons of age or prevailing circumstances.

In the case of surgical derived offspring, the donor dam is euthanased at the end of the surgery without regaining 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 animal type)?

This Project Licence would allow a number of farmed species (sheep, pigs and cattle) to be blood sampled and returned to stock and is expected to be of mild severity for all animals. (90% of all project animals)

For the delivery by Caesarian section under general anaesthetic (sheep, pigs) of infection-free offspring this is considered Non-recovery for all animals (10% of all project animals).

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Blood can only be obtained from the actual animal. Currently, different species are required on this licence depending on the proposed use of the blood products e.g avian blood is required for haemagglutination-inhibition assays. Some donors are chosen as the animal is the natural host of the disease being investigated. The subsequent scientific work is generally lab-based and often provides a platform for *in vitro* work replacing the infection of the live animal.

Live neonatal animals, in the numbers required, can only presently be obtained from an adult female animal.

Which non-animal alternatives did you consider for use in this project?

Scientists at the establishment endeavour to use alternatives to live animals and blood supplied will often be used in *in vitro* models.

Attempts at developing alternative serological assays, such as ELISA, have been unsuccessful and currently using goose blood in Hemagglutination-inhibition assays (HIA) is the only assay available for Louping ill virus (LIV) surveillance and disease diagnosis in the UK.

Where possible, colostrum-deprived animals can be substituted in place of caesarian-derived animals and this is done under a different Project Licence.

In some cases it is possible to source antibody negative animals for particular diseases from the field which can then be reared conventionally, which removes the need for caesarian-derived SPF animals.

Why were they not suitable?

Many immunological assays require specific cells from within the blood to cause a detectable reaction with the pathogen.

Where it is impossible to confirm an animal has not been exposed to a disease, as in the presence of a ubiquitous pathogen or where no diagnostic test exists, it may be necessary to produce an animal of known health status, under these circumstances.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the historical frequency that samples are requested and the need to rotate animals to ensure sample frequency is acceptable to local guidance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior to the surgical delivery of gnotobiotic and SPF animals, a biometrician will review and advise on the number of neonates required for each experiment. Pregnant animals are, where possible, scanned in advance using ultrasound to determine the number of foetuses. The number of dams used is kept to a minimum by using dams with high numbers of foetuses.

The use of SPF lambs provides reduced experimental variation than would lambs in standard housing, thereby providing a refinement to the experiment and reducing experimental group size and contributing to the goals of the 3Rs.

Use of SPF or gnotobiotic caesarian-derived animals can actually reduce the overall number of animals used, since it is not uncommon for conventional vaccine studies to collapse because of seroconversion in the negative control group.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All proposed procedures to be carried out under the authority of this licence are assessed by the AWERB of the Institute. Requests for these materials and animals are co-ordinated so that the minimum number of animals are required.

The minimum number of each species required for blood sampling are kept so that the limitations on frequency and volume are not exceeded while normal demand is met.

Although the blood samples are essential for our research, many of the research groups require them only periodically and it would not be cost effective to maintain sole-access animals for each group. By accessing samples through this project, this spreads availability over a wide range of research projects, thereby reducing the number of animals required. If every research lab maintained their own group of sheep and cattle, there would be significantly more animals required overall.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 species chosen are those required by the scientists for their particular projects and are often the natural hosts of the disease under study. Trained statisticians are available to provide independent advice on experimental design. All projects require approval by the AWERB, which includes a biometrician.

Trained, experienced, licenced staff are used to collect the blood samples. Training records are audited. Excellent farm animal handling facilities are on site. Stockmen assist with correct restraint and report any concerns regarding competency of phlebotomist.

Gnotobiotic and SPF surgical deliveries, to produce animals of known health status, are performed by trained, experienced, licensed Veterinary Surgeons, under general anaesthesia. These are non- recovery procedures.

Why can’t you use animals that are less sentient?

Healthy, mature animals are the most suited to donate blood.

Dams undergoing surgical delivery are terminally anaesthetised and performed as a non-recovery procedure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal technicians are trained and experienced in animal restraint. The regular blood donors become accustomed to the minor procedures and experience minimal stress. Animal technicians monitor the animals at regular intervals and seek veterinary attention for any animals of concern.

The delivery of SPF animals has been developed and refined over the years to ensure good anaesthetic and aseptic technique.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Percie du Sert, N., Hurst, V., Ahluwalia, A. et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. BMC Vet Res 16, 242 (2020). https://doi.org/10.1186/s12917-020-02451-y

Guidance from NC3Rs and Norecopa will be followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

NC3Rs, Norecopa and LASA Website. The Establishment 3Rs Group.
Relevant training courses and communication with colleagues working in the field.

161. Effects of Anaesthetic and Other Related Agents on Organ Injury and Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Anaesthetic, Organ injury, Cancer, Molecular mechanism, Therapy

Animal types	Life stages
Mice	adult, embryo, pregnant, neonate, juvenile
Rats	adult, embryo, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to investigate the impacts of anaesthetic and other related agents on organ injury 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 early findings of studies we have been doing over a few years are promising but more studies are needed in order to develop our ideas to benefit patients. This project focuses on four different areas with the aim of improving patient outcomes:

Kidney ischaemia-reperfusion injury

Kidney transplantation

Hypoxic-ischaemic encephalopathy (HIE) caused by a shortage of oxygen during birth leading to brain injury

Use of anaesthetics in cancersurgery

Firstly, acute kidney injury (AKI) remains a formidable medical challenge worldwide. AKI has become the focus of intense epidemiologic, clinical, translational, and basic science research. This focus is well-deserved given an incidence of 21.6% for all hospitalized adults worldwide, mortality of 23.9%, and an association with the progression of chronic kidney disease (CKD) to end-stage renal disease (ESRD). AKI has numerous etiologies that all result in similar structural and functional outcomes. It is increasingly recognized that AKI is a major contributor to the development of CKD. CKD affects approximately 10% of the world's population and places a significant burden on economy, society and healthcare worldwide. Acute kidney injury caused by ischaemia-reperfusion injury is a significant clinical problem in cardiovascular surgical procedures and kidney transplantation. With more surgical procedures being performed within an aging population, the incidence of acute kidney injury associated with ischaemia-reperfusion after surgery is increasing; this has a high rate of mortality and morbidity. Furthermore, the cost of managing patients with these complications is a huge burden on the NHS. Therefore, investigating the new underlying molecular mechanisms of kidney ischaemia-reperfusion injury and developing novel therapeutic strategies to protect the kidneys are urgently needed.

Secondly, more than 9,000 people in the UK need an organ transplant to save or improve the quality of their lives; most are awaiting kidneys. In 2007, more than 6,000 patients were on the waiting list for kidney transplantation. Donor organs are highly precious resources, but transplants can fail completely or not function as well as they should. Our research will look at new ways of reducing transplant failure and our findings could be extended to all organ transplantation where similar conditions apply. Kidney transplantation is the best treatment for patients with end-stage kidney failure and gives the best results, good quality of life and cost savings when compared with other treatments such as dialysis. There is a severe shortage of donated kidneys and efforts are being made to increase availability by extending the range of people who can donate organs, including non-living donors. Our previous research has shown that the use of the new anesthetic gas xenon before, during or after organ removal and transplant can greatly improve chances of successful transplantation.

Thirdly, HIE is a devastating condition caused by a shortage of oxygen at birth and remains a major cause of sudden death and long-term nerve disease in infants and children even in the developed world. This type of brain injury is currently seen in between 1 and 6 per 1,000 live births in the developed world with approximately 30% of infants who survive developing life-long conditions including cerebral palsy, learning disabilities, epilepsy and mental retardation. The financial and emotional cost of caring for those affected by HIE is considerable due to the severity and lifelong nature of the handicap. In addition, it is common for legal action to be taken by families of those affected, with claims costing the NHS about £300m a year and in 2006, it was estimated that there were outstanding claims of £5 billion for HIE related cases. There are currently limited methods that can be used to reduce the likelihood of HIE related brain injury, so it is a major priority to develop new methods.

Finally, cancer is a major public health problem in the world causing disease and death. Despite better treatments for cancer over recent decades, the chances of cancer coming back after surgery are still high. It has been accepted that surgery itself can lead to cancer spreading to other parts of the body. The effect of anaesthetics has not been thoroughly investigated, despite some studies indicating that the choice of anaesthesia could lead to better patient outcomes after surgery. Some recent studies we have done in the laboratory have led us to believe that there are clear reasons for this at the small scale.

What outputs do you think you will see at the end of this project?

The major benefits expected to be obtained from this project are in new information on some of the most commonly used anaesthetic agents and pain relief medicines. While most of this work is still in the basic science field, much of the information will be useful to other researchers and an important knowledge source for clinicians in relation to their work. We hope to publish our work in well-respected scientific journals. The impact of our research outputs are as follows:

Kidney ischaemia-reperfusion injury

Acute kidney injury (AKI) such as ischaemia-reperfusion injury remains a formidable medical challenge worldwide. Unfortunately, no effective treatment strategies are available due to an insufficient understanding of the underlying mechanisms. If our research outcome is positive, large animal studies and clinical trials can be initiated subsequently for enhancing patient recovery.

Kidney transplantation

As a mainstream therapeutic method, kidney transplantation has drastically transformed the health quality of patients suffering from end-stage renal failure. Success in renal transplantation is promoted by introduction of powerful immuno-suppressive medication. However, the long-term outcome of the transplanted kidney is still far from ideal. A high priority in kidney transplantation research, therefore, lies in the development of reno-protective strategy, which confers a long-term benefit in graft survival. We have been working in developing novel renoprotective strategy against early graft injury and later graft lost and this will ultimately enhance the recipient survival and increase the number and quality of donor organs.

Neonatal brain injury

Perinatal hypoxic-ischaemic brain damage due to lack of oxygen to the brain during birth remains a major cause of mortality and morbidity. It is crucial that strategies are developed to minimise the long- term neurological sequelae of fetal hypoxia-ischaemia during birth. Our research will lead to development of novel neuroprotective therapies against neonatal hypoxic-ischaemic encephalopathy, which will reduce the impact of this injury on the neonates.

Surgery, anesthetics and cancer

Cancer remains the leading cause of death worldwide. Surgical resection is a commonly used method for patients with solid tumors. However, tumor metastasis after cancer surgery often occurs, which is mainly responsible for deaths in cancer patients. Our research will improve the understanding of the molecular mechanisms of tumor growth,

angiogenesis, metastasis and chemo-resistance and more importantly, the factors which promote cancer recurrence after surgery.

Who or what will benefit from these outputs, and how?

Our research will be published once completed, and available to other researchers working on similar themes. The way that many of these anaesthetic agents work is through known routes in the body, which may lead to ideas on the possible actions of similar drugs. Other researchers may use our findings to identify other drugs which give useful results. In the longer term, many of the anaesthetics we use may be able to be used for a wider range of patients, including the most vulnerable. For example, if one anaesthetic is found to increase spread of certain cancers, planning the best treatment for patients could take this into account. It may be possible to change practices in operating theatres and hospitals to improve outcomes in future.

Additionally, recent findings suggest that Argon may have great protective benefits, with the added bonus of being an extremely cheap commodity. Xenon is currently undergoing clinical trials for the protection of the nervous system in newborns, a research field our group has been involved in and published data on. There is very little published data on Argon, so any findings from our research would greatly increase knowledge of how this agent works.

How will you look to maximise the outputs of this work?

The proposed projects are likely to be published in highly respected scientific journals and we will present our research data in the national and international conferences for promoting the dissemination of new knowledge to the general public.

Species and numbers of animals expected to be used

- Mice: 3500
- Rats: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project hopes to develop and test a range of interventions in different patients' illness, especially anaesthetic agents. If these proposed inventions or strategies are effective, then they can be introduced to clinical practice for patients' benefits. As most people will be exposed to anaesthetic agents at least once in their lives, research into the true potential of these drugs to cause any lasting benefits or harm need to be carefully considered. This project aims to determine in a number of scenarios which anaesthetic agent may be the most appropriate to use to reduce adverse outcomes for patients.

Mice and rats are used for different protocols in this PPL (mice for kidney ischaemia-reperfusion injury, rats for transplantation, both mice and rats can be used for neonatal asphyxia study and SCID and nude mice for cancer study). Different life stages will be chosen to simulate the disease or injury occurring in humans. For examples, neonatal

asphyxia in rats/mice is used to need to mimic hypoxia events in early life in humans, so specific postnatal days are chosen to correspond to specific ages in human. Rodents share highly similar mammalian anatomy, physiology and immune system with human. Laboratory rodents are inbred with fast reproduction rate and identical genetic background, allowing experiments to be completed within relatively short period of time with very good reproducibility. Genetic modification is easier in mice and rats will allow easier surgical manoeuvre.

Typically, what will be done to an animal used in your project?

The protocol number and major procedures to be performed on animals under this licence are:

1: Renal Ischaemia
Pre-treatment of pharmacological agents or hypoxia. Under general anaesthesia, laparotomy with or without clamping of renal pedicle or Laparotomy with unilateral clamping of one renal pedicle (maximum time 45 minutes) combined with unilateral nephrectomy of the second kidney (to prevent compensation) or laparotomy to clamp renal pedicle vasculature bilaterally (maximum time 25 minutes).

2: Kidney Donor

Pre-treatment of pharmacological agents or hypoxia. Under general anaesthesia, unilateral nephrectomy and harvest kidney graft.

3: Kidney Transplantation

Under general anaesthesia, A kidney organ that has been harvested from a donor animal in protocol 2 will be used for transplant. Unilateral nephrectomy is performed in the recipient animal, followed by single kidney transplant with end-to-end anastomosis of renal artery, vein and ureter, followed by removal of second kidney. Post-treatment of pharmacological agents.

4: Perinatal asphyxia in dam

Pre-treatment of pharmacological agents. Dam will then be killed by schedule 1 method and laparotomy performed following confirmation of death. One uterine horn is clamped for a periods of time to induce hypoxia and placed in temperature controlled saline for period ranging from 5 to 30mins. Foeti in remaining horn are delivered via caesarean section for immediate resuscitation.

5: Perinatal asphyxia in pups

Pups come from harvest of uteri of gravid dams in Protocol 4. Animals after delivery (at any age) will be exposed to hypoxia (8% O2) in a purposely built hypoxic chamber for up to 2 hours. After a period of approximately 6 weeks, animals will undergo any of the behavioural studies.

11: Cancer growth, metastasis, diagnosis and therapy for solid cancer model

NSG or SCID mice will undergo injection implantation of a cell suspension of cancer cells under anaesthesia, such as A549 and Caco-2, either subcutaneously, or via tail vein or

intraarterially (surgical exposure of carotid artery). Anaesthesia is then maintained for up to 2 hours. Vehicle control will be administered in some animals.

12: Cancer growth, metastasis, diagnosis and anaesthetics for leukaemia model

Animals will undergo implantation of a cell suspension of cancer cells through tail vein injection or be given injection of chemicals. Animals are treated with anaesthetics agents. Anti-cancer drugs (e.g. Cytarabine Ara C) are given to mice intravenously or intraperitonially.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of adverse effects associated with this license are mild in nature and are not expected to be seen, such as following simple anaesthetic gas exposure. Some adverse effects may be seen following surgical procedures and if cancer or leukaemia are brought about in the animal, but we will follow strict sterile techniques and check animals daily so that adverse effects should be rare. We have detailed instructions for the care and monitoring of animals and animals will be humanely killed if they show an symptoms unexpected severity. Actual severity will be recorded for each individual animal to allow us to closely monitor the progression of each protocol and ensure close tracking of animal welfare, as well as experimenter competence.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All procedures are either mild or moderate severity. All animals (100% of mice or rats which undergoing these procedures) will experience the severity level. All animals will be killed after experiments in a humane manner.

1: Renal Ischaemia
Severity category: Moderate
Percentage of mice or rats undergoing this severity: 100%

2: Kidney Donor
Severity category: Moderate
Percentage of mice or rats undergoing this severity: 100%

3: Kidney Transplantation
Severity category: Moderate
Percentage of mice or rats undergoing this severity: 100%

4: Perinatal asphyxia in dam
Severity category: Moderate
Percentage of mice or rats undergoing this severity: 100%

5: Perinatal asphyxia in pups
Severity category: Moderate
Percentage of mice or rats undergoing this severity: 100%

6: Standard Superovulation
Severity category: Mild
Percentage of mice undergoing this severity: 100%

7: Standard Generation of founders
Severity category: Mild
Percentage of mice undergoing this severity: 100%

8: Standard ICL Embryo recipients
Severity category: Moderate
Percentage of mice undergoing this severity: 100%

9: Standard Vasectomy
Severity category: Moderate
Percentage of mice undergoing this severity: 100%

10: Standard ICL Breeding and maintenance of GA animals
Severity category: Mild
Percentage of mice undergoing this severity: 100%

11: Cancer growth, metastasis, diagnosis and therapy for solid cancer model Severity category: Moderate
Percentage of mice undergoing this severity: 50-100%

12: Cancer growth, metastasis, diagnosis and anaesthetics for leukaemia model Severity category: Moderate
Percentage of mice undergoing this severity: 50-100%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have considered whether we could avoid using animals, but there are no alternatives which can reproduce the whole body responses that we will be investigating. Active efforts have been made to achieve the best replacement, refinement and reduction procedures possible, with advice from the NVS and NACWO.

Which non-animal alternatives did you consider for use in this project?

Extensive laboratory work will be carried out before beginning experiments with animals to work out the best dose levels for the substances we are investigating.

A number of resources (such as the European Union Reference Laboratory for alternatives to animal testing) were looked at during our planning stages to see if there were any different ways of doing this research but due to the nature of this work, none were suitable.

Why were they not suitable?

Due to the complex nature of the systems studied, such as nerve damage or organ inflammation, a whole animal is needed. As our studies address clinical issues it is necessary to observe whole body responses to any given treatments as well as to ensure no unexpected side-effects occur in animals. Wherever possible, studies of drugs will be carried out in the laboratory rather than on 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?

Statistical analysis will be used to establish the minimum number of animals needed for meaningful results. The design of our experiments and analysis of the results have been and will be further discussed as projects progress. We will aim throughout to keep numbers of animals to the minimum necessary for our studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We carried out extensive work in the laboratory first to help us identify the most realistic treatments to investigate in our animal work, for example live imaging, along with statistical calculations. Careful management of breeding in order to provide pregnant females and ‘sham’ pregnancies with which to compare these, will ensure that no animals are bred unnecessarily.
We aim to get the maximum amount of information from the minimum number of animals.

The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines will be followed throughout the project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be carried out to optimise the number of animals. Laboratory studies, such as growing cells in dishes, will be used to establish the mechanisms whenever it is possible.

Sharing of animal tissues will be actively considered based on the availability of tissues in other research groups. Breeding will be carefully controlled.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models used for this project are listed below:

Rodent kidney ischaemia-reperfusion injury model

Acute kidney injury caused by ischaemia-reperfusion injury is a significant clinical problem in cardiovascular surgical procedures. We use the rodent model of ischaemia-reperfusion injury to investigate the underlying molecular mechanisms and protective strategies. The surgery is performed under general aneasthesia and analgesia is provided to reduce the pain and suffering. The animals are monitored on the daily basis after surgery to ensure adverse effects are minimized.

Rodent kidney transplantation model (donor and recipient)

The rodent kidney transplantation model is used to assess the protective effects of proposed therapeutic strategies. For the donor surgery, we have considerable experience of single kidney removal. Stress to the kidney donor is reduced by carrying out much of the experimental treatment on the kidneys after they have been removed from the body. This reduces the degree of handling and treatment for donor animals. For the recipient surgery, we conduct the artery, vein and ureteral anastomosis under general anesthesia. In addition, analgesia will be provided to reduce the pain. The animals are monitored on the daily basis after surgery to ensure adverse effects are minimized.

Rodent neonatal brain hypoxia model
This rodent model involving both mothers and newborns will be carried out to investigate the treatment methods on the neonatal brain hypoxia injury. All treatments given will be chosen to give the least stress possible to the mothers. Weighing pups and handling procedures will be reduced as much as possible, usually only three times during the first week so as not to stress the mothers and to ensure there is no rejection of surrogate pups. All animals used for evaluation of the nervous system, such as in a water maze, will be checked beforehand to ensure there is no physical defect which may affect their ability to perform behavioral tests, such as swimming.

Animal tumor xenograft model

The tumor xenograft model is used to investigate the cancer development and treatment strategy. It is necessary to use nude mice for our cancer studies, and they are prone to infection. These animals will be carefully monitored and when measuring tumour development using imaging techniques, will only be used for one of the methods of imaging. Limits on the number of sessions for each individual will be set to minimise stress.

Why can’t you use animals that are less sentient?

Laboratory rats and mice provide ideal animal models for biomedical research and comparative medicine studies because they have many similarities to humans in terms of anatomy and physiology. The use of rodents also provides advantages related to the

wealth of genetic information available to scientists. The identification of genes responsible for organ injury and cancer provides an excellent example of the utility of rodent for studying human organ injury and cancer, and their treatment strategy. Using comparative genomic techniques, identification of a gene responsible in the rodent allows the equivalent gene to be identified in human. Rats are the preferred rodent model for cardiovascular and transplantation surgery where their larger size is an advantage, especially for facilitating surgical procedures and other types of testing after surgery. The rodents are commonly used for behavioral studies because they are much more social than other animals and their behavior better mimic behavior seen in humans. Animals that are less sentient, such as fish, do not have the above mentioned advantages in the rodent models and therefore can not be used as the specific scientific models in our studies. Animals at a more immature life stage can not be used in our project as organ injury or cancer occur mainly in the mature life stage in humans. Animals that have been terminally anaesthetised do not serve the purpose in our studies as we intend to explore the long-term effects of proposed treatment on the animals, which is particularly important for the prognosis and recovery of our patients.

The rodent models used in this project are firmly established in our lab and accepted by the scientific community. In addition, mice which are genetically altered in the way we need are easy to obtain and makes them the most appropriate species for our work. The mice and rats are raised in controlled, pathogen-free environments, provided by our housing facilities. and the effect of environmental factors can be highly controlled.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Based on our previous work, along with discussions with veterinary staff, the projects have been designed in a way that minimises suffering of animals and to cause the minimum severity possible. We will monitor severity during all our experiments and raise any concerns immediately with the NVS, NACWO and/or Home Office.

Animals will be housed and maintained in groups, where possible, and given environmental enrichment and free access to food according to best practice as advised by the animal technicians. Each experiment has a strict list of possible adverse effects, humane endpoints and procedures to follow when there is any doubt over the welfare of an animal. Wherever possible the mildest pre- treatments and surgical methods will be used to obtain the most relevant data. Where a more harmful procedure must be followed, each animal will be closely monitored to ensure their health and welfare, and the welfare of the animal will be placed before scientific outcomes. In some protocols, the nature of our studies investigating anaesthetics and pain relief may even reduce pain and suffering the animals might experience.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines to ensure experiments are conducted in the most refined way. We also will follow aseptic surgery guidelines,
established in our animal facilities and the guidelines for cancer studies in rodents, which is essential for the care of the rodents with tumor xenografts.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The national center for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) will provide latest advances in the 3Rs. We will stay informed about their new policy and knowledge and implement these advances effectively in our projects. In addition, we will be advised by the local 3Rs manager for effective and timely response and implementation.

162. Immunity to ectoparasites of ruminants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

vaccines, ectoparasite, livestock, ruminants, immunity

Animal types	Life stages
Sheep	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The specific aims of the project are:

To understand the nature of the protective immune response which is induced by exposure to, or administration of vaccines against, the ectoparasitic mite, *Psoroptes ovis* in sheep.

To use this information to improve the effectiveness of vaccines such that they will provide an important tool in the control of infestation with *Psoroptes ovis* (sheep scab or psoroptic mange).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 vaccine that is being developed in the project will directly benefit livestock by protecting them from parasitic disease. This benefits both the welfare and productivity of the vaccinated animal but also reduces reliance on synthetic chemicals, adding to global food security and reducing environmental contamination. In addition, the parasite which is being investigated here has close relatives, which have negative health impacts on humans and other animals. i.e. scabies mites (*Sarcoptes scabiei*) and the house dust mite (*Dermatophagoides farinae*).

What outputs do you think you will see at the end of this project?

New prototype vaccine for further development to commercial products

New information on the host: parasite interaction

New information on vaccine and adjuvant effects on the immune system

Multiple scientific publications and lay articles on livestock vaccines

Who or what will benefit from these outputs, and how?

Short term beneficiaries will be researchers working in the fields of vaccinology and ruminant immunology through the availability of new information on the host:parasite interaction, immunology and vaccinology which will accelerate their own research programmes. Longer term beneficiaries include stakeholders in the vaccine and livestock industries through the production of new commercialisable tools for animal health and welfare. Societal benefits can also be seen through the reduction on reliance of injectable macrocyclic lactones and organophosphate dips with the attendant environmental and safety concerns that these bring.

How will you look to maximise the outputs of this work?

The applicants have a long track record of collaboration and effective knowledge exchange and outputs will be maximised through the following approaches:

Scientific Audiences: The results of our investigations will be published in open access journals appropriate for subject matter, 3Rs and scientific impact. All manuscripts will follow the ARRIVE guidelines [Kilkenny et al., 2010. PLoS Biol 8(6): e1000412] to promote accurate reporting appropriate for 3Rs initiatives.

During the course of the project we will attend scientific conferences (e.g. the World Association for the Advancement of Veterinary Parasitology Conference) to communicate progress and to emphasise the focus on scientific excellence in this project, highlighting crossovers in disciplines. The licence holder will attend the British Society for Parasitology Annual Spring meeting each year to communicate these aspects. The licence holder is an active member of the BBSRC's International Veterinary Vaccinology Network and will attend their annual meetings to disseminate the approach and results (both successful and unsuccessful) to this community. The licence holder interacts with a large number of researchers through formal networks, which promote co-operation and multidisciplinary networking between scientists and stakeholders. In addition, as objectives are successfully completed we will directly communicate the emerging technology by email and/or Microsoft Teams meetings to the research groups most likely to benefit from it.

Educational and Public: The licence holder teaches undergraduate and postgraduate students in parasitology and will use these opportunities to promote the outputs and principles of this project. For dissemination to the wider public, the applicant will attend the Royal Highland Show in each year of the grant, to communicate our results, their impact and context to members of the general public. The impact of the project will be made available on the host institution website as a resource for the public, policymakers and Government stakeholders in the food, animal health, environment and rural sectors.

Species and numbers of animals expected to be used

- Sheep: 950

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used in these protocols are the natural hosts for the parasite under study. The life stages of the ruminants used are the most susceptible to this parasite and also the stages for which the economic impact of infestation isthe highest.

Typically, what will be done to an animal used in your project?

Animals in this project will be administered vaccines by the most appropriate route (this may be injections or sprays for example) and then infested with *Psoroptes ovis* mites using our standard challenge infestation. Typically animals would receive 3 injections of the vaccine, 2 weeks apart and would then be challenged with the mites for a maximum period of 6 weeks in experimental settings.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects on the animals are expected to be moderate. Infestation of the skin with *Psoroptes ovis* is characterized by exuberant yellowish scabs, additional signs include restlessness, pruritus, scratching, yellow-stained fleece, wool-loss, head tossing, bleeding wounds and loss of condition. In particular, the disease can cause considerable irritation or pain, or both. Typically these effects would be for the duration of the infestation, so a maximum period of 6 weeks in an experimental setting. However, we have extensive experience on the course of infestation following experimental challenge and, at termination of the experiment the area of lesion is expected to be less than 20% of total body surface area. The injection of recombinant *P. ovis* proteins as part of the vaccine is considered unlikely to cause any adverse reaction, although animals will be carefully monitored during and immediately following these injections. Quil A adjuvant has been widely employed by ourselves and others in vaccines in sheep, without any adverse reaction. Infestation of sheep scab mites will be monitored closely.
Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals infested with Psoroptes ovis would be expected to suffer a maximum of a moderate degree of severity. This will be controlled by limiting the number of mites used for each application to ~100 mixed stage mites. Animals will be monitored, and infestations will only be allowed to proceed until a maximum of 20% of the body surface is affected or for a maximum of 6 weeks duration.

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 parasites being studied in the project are "obligate" parasites. This means that they cannot survive off of the host animal. We must therefore use host animals (sheep) to maintain these parasites before and during vaccine trials.

Which non-animal alternatives did you consider for use in this project?

Currently there are none as the mite *Psoroptes ovis* cannot be maintained off of the live ovine host. However, it is our intention to continue to work towards developing an off-host feeding and maintenance system for *P. ovis*. Previous attempts to develop such a system have successfully maintained mites in vitro for a period of 3 weeks, however the mites were unable to progress through their entire life cycle in this time (egg, larvae, protonymph, tritonymph, adult (male and female)). Future research in our group will continue to seek alternatives to replace the need to use live animals for these studies with a focus on developing an efficient off-host rearing system that is able to progress the mite life cycle from eggs to adults.

Why were they not suitable?

Currently no suitable alternatives exist but we will continue to work towards developing such a system 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?

These estimates are based on the numbers of animals used in previous studies over the last ~10 years and have been optimised through collaboration with statisticians reviewing data from our previous experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior to each experiment, we review data from previous similar experiments, estimate the levels of effect of any treatment that we want to be able to record and collaborate with expert statisticians to determine the minimum numbers of animals required to accurately determine treatment efficacy

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Maintaining good animal health throughout every experiment to avoid animal numbers reducing from disease which is not related to the disease under study. In addition, wherever possible we will make samples taken from live animals and any tissues from any euthanased animals available to other researchers for their use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The species being used (sheep) is the natural host for *Psoroptes ovis* and also the target species for the vaccine being developed, so are the most appropriate model species to be employed here. Vaccination and challenge of sheep consists of 3 immunisations with the candidate antigen(s) in adjuvant (QuilA) two weeks apart and animals are then experimentally challenged with a carefully controlled mite infestation. During infestation and vaccine testing, sheep are routinely monitored by veterinary staff and are treated with veterinary medicines if required, based on clinical symptoms.

Why can’t you use animals that are less sentient?

The mite, *Psoroptes ovis* has adapted to surviving on lambs and ewes and has a relatively long-lasting lifecycle (several weeks) as such the use of terminally anaesthetised animals would not be practical. In addition, as previous infestations can result in a degree of protective immunity, we need to ensure that the animals used in the trials are naive for sheep scab with no history of infestation, which is easier to guarantee in younger animals with a known history.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Monitoring post procedures will identify any areas which require refinement. For example, animals are closely monitored following vaccination and, if any of the compounds administered cause pain, appropriate analgesia will be administered

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Where appropriate, the World Association for the Advancement of Veterinary Parasitology guidelines for best practise will be followed https://www.waavp.org. In addition, the ARRIVE guidelines 2.0 will be followed to allow the experiments to be performed in the most refined way and the publication of the data in the most appropriate form. https://arriveguidelines.org/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The host institution holds numerous NC3R research grants and has regular contacts with this body. In addition, we have an active 3Rs committee, which provides advice to project and personal licence holders. Any relevant advances in the 3Rs will be implemented into any of the protocols where appropriate.

163. Immunity, Inflammation and Tissue Function

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Inflammation, Immunity, Homeostasis, Macrophage, Tissue Damage

Animal types	Life stages
Mice	juvenile, neonate, pregnant, adult, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is: to understand how immune cells behave within and interact with their environment during health and conditions of immune challenge/disease; and to understand the molecules and pathways that regulate these activities. The objectives of this project are: to better understand the processes that drive appropriate inflammatory responses and also maintain normal tissue function; the overlap between these processes; and, how one process, when dysregulated or modulated, may negatively or positively impact on the other.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The immune system plays a fundamental role both in normal tissue function and in the progression of number of diseases: including cancers; atherosclerosis; autoimmunity, such as arthritis; and neuroinflammatory conditions such as Alzheimer's disease. Inflammatory responses can be both protective (eg. combatting infections or repairing injury) or damaging (e.g. excess collateral tissue damage and fibrosis). Understanding the regulators of inflammatory processes will provide novel insights into therapeutic

opportunities for promoting a healthy resolution to inflammation with minimal tissue damage.

What outputs do you think you will see at the end of this project?

A holistic view, should identify novel therapeutic approaches to the regulation of inflammation and restoration of tissue function. Hence as a primary benefits we anticipate the further development of potential targets that regulate inflammation and tissue repair. The primary output of this project will development of new knowledge and publications.

Who or what will benefit from these outputs, and how?

In the short-term, this project will establish new understandings about immune mechanisms in health and disease. In the medium-term, this knowledge may help redefine physiological processes and provide new insights into tissue dysfunction and disease processes. In the longer-term our objective will be to exploit this new knowledge, where possible, for better outcomes for human disease through ongoing and new collaboration with industrial and clinical colleagues.

How will you look to maximise the outputs of this work?

The applicant is already part of national networks and has active collaborations with industrial and clinical groups. All new knowledge will be published in peer-reviewed journals, with pre-prints archived in dedicated freely-accessible repositories to accelerate knowledge dissemination to all interested parties. Opportunities for exploitation of the knowledge, for example, more detailed analysis of disease mechanisms with therapeutic objectives, will be explored with our collaborators and additional interested parties.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project use mice. A mammalian species must be used because of the complexity of the innate and acquired immune systems in mammals. Mice are the most appropriate species because they are the species with the lowest degree of neurophysiological sensitivity in which genetic manipulation can be reliably and reproducibly achieved. Animals will be studied at all stages of life. The majority of interventions (e.g. injections) will occur in mice over the age of 4 weeks. A small number of neonatal mice may be studied to observe tissue development.

Typically, what will be done to an animal used in your project?
A significant number of animals will be used in breeding colonies to maintain lines of genetically- altered, or unique mice. Many mice will be studied without further treatment, for example, as a source of normal tissues or cells, or for study of normal tissue function. The vast majority of remaining mice that will be administered substances by injection via the

appropriate routes or will be treated orally (altered drinking water, food or by gavage). A small number of cases will involve surgery, for example to use a minipump for continuous drug delivery to negate the need for multiple injections. In another small number of cases, the mice may be exposed to radiation to enable bone marrow transplantation.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the experiments will be mild in severity. They will involve one, of a few stimuli, that evoke a self-resolving transient inflammatory response, which will be studied with various interventions to understand the processes occurring. Some procedures will involve stimuli of moderate severity, such as surgery or experimental infection, where clinical signs of adverse effects will be more evident or longer lasting. In some cases we will study mouse models of spontaneous chronic disease development, to understand how they develop and how we may intervene. In all cases, the presence of adverse effects is specifically monitored for and the mice will be humanely killed if they reach specific humane endpoints.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of mice will experience only mild or sub-threshold severity. A minority of approximately 5-10% may experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are modelling the changes in immune response and in tissues that occur over time. These are not localized to individual tissues, but are regulated by systemic changes, involving, for example, the migration of immune cells to peripheral and immune tissues.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we have developed in vitro systems for the addressing our specific questions, for example, we routinely use and produce cell line models as a direct replacement. These are both pre- existing cell lines, cell lines we make ourselves from the mice and human induced pluripotent stem cells. These can in some instances be grown in co-culture to better approximate the physiological systems we aim to model.

Why were they not suitable?

Cell lines do not display all the characteristics of the cells found *in vivo* and whilst cell culture techniques have dramatically improved, the microanatomy, distribution of multiple cell types and the presence of many diverse cell:cell and cell:extracellular matrix interactions found *in vivo*, which play complex roles in the regulation of immune cell activation and regulation, cannot be replicated *in vitro*. Current modelling approaches also do not replicate this *in vivo* complexity necessitating the use of animals. We do, however, still use cell models whenever possible to model aspects of immune responses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This project will require the in house breeding of several lines of genetically modified animals. These will need to be maintained for the duration of the project and will represent the bulk of the animals used during this project. Estimates for the maintenance of minimal numbers of each line and the subsequent generation of offspring are included in this estimation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Every experiment conducted is reviewed for statistical validity. Where sufficient data is available power analysis will be conducted using freely available tools (e.g. G*Power 3.1 and the NC3R's experimental design assistant) to determine the appropriate experimental sizes required to generate meaningful data. In the absence of sufficient data, recognized approximations for experimental size will be used (e.g. the Resource Equation) or smaller pilot experiments will be conducted first to determine the variability of the experimental parameters enabling calculation of the appropriate experimental size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We aim to keep breeding colonies to the smallest size necessary, cryo-preserving lines when it is clear they will not be used for a period of time. We review the literature and conduct pilot studies to establish the variation within experiments and also try to establish alternative experimental readouts with less variation that could be used as an alternative and would require fewer animals. Our team won a local 3Rs prize for our tissue sharing initiative and we are in discussions with a commercial partner to establish the process not just institution-wide, but in multiple 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are primarily using genetically-modified mice to study aspects of the immune system. Many mice will receive no further intervention. The majority of the remaining mice will receive one or a small number of substance administration via appropriate routes. In some cases, recurrent administrations are needed and in a small number of animals surgically implanted osmotic minipumps (or similar) may be used in place of repeated administrations. More fundamental approaches include use of refined injections (for example, using the smallest gauge needles and limiting injection volumes as much as possible); use of refined routes (for example we use pipette feeding instead of oral gavage whenever possible, which the mouse becomes accustomed to and doesn't have the potential complication of oesophageal damage caused during gavage); use of reduced doses where scientifically appropriate to limit the potential for or intensity of adverse effects and events. All these approaches help to refine the use of animals in our research ensuring the lowest possible level of pain, suffering or distress caused.

Why can’t you use animals that are less sentient?

Our primary aim is to study the immune system in adults in a multicellular and tissue-specific manner. This requires mammals whose tissues development and physiology resembles that of humans. The use of mice enables the use of genetic modification to enable intricate dissection of disease mechanisms and mice are the mammals with the lowest neurophysiological sensitivity that can be reliably genetically modified. Lastly, the need to study inflammatory and immune processes necessitates that living animals be studied over a period of time.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have introduced a number of refinements during the course of our work, including the use of pipette feeding in place of oral gavage, the use of smallest gauge needles and injection volumes permissible for reliable data. Additionally, we routine use the lowest doses of drugs and other substances to achieve the required biological effects, with minimal adverse effects. In cases of surgery, we would consider deliver of prophylactic pain relief and hydration, always under the guidance of the named veterinary surgeon.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We routinely derive best practice instruction from publications such as Wolfensohn and Lloyd with regard to recommended administration volumes, routes and methods. We also monitor resources, such as those managed by the NC3Rs. Every experiment is reviewed prior to commencement using a proforma that considers the protocol, including its statistical validity and adherence to the requirements of the license. In addition to the current statistical considerations where we consider reduction, we will add a comment on the review of the 3Rs into this documented process, whereas previously it would have been considered, but less explicitly documented at this planning step.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are able to keep up to date with the 3Rs via several mechanisms: We have a programme of sharing 3Rs knowledge run locally, where new innovations and approaches are shared with other staff members; A member of the NC3Rs sits on our ethical review panels and offers advice on 3Rs at all stages from project licence development to ad hoc protocol development; we are also able to monitor national developments via the NC3Rs online resources and newsletters (developments of which are delivered both personally and via internal institutional communications). Our research centre is part of a multicentre national research institute and we have 3Rs champions who meet to share best practice between the centres. 3Rs advances can take many forms, from minor refinements to protocols, to changes in the statistical analysis of data and primary outputs that adjust the number of animals required for meaningful data.

164. Immunity and Resistance to Disease in Cattle

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immunity, Vaccines, Disease, Tuberculosis

Animal types	Life stages
Cattle	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand the immune responses that are important in cattle that are vaccinated against, or infected with, important diseases that affect their health and welfare.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diseases in cattle have a significant impact on animal health and welfare, and impact on the economy. Two important diseases caused by Mycobacteria are bovine tuberculosis (TB) and Johne’s disease (a gut condition). The bacteria that cause these diseases can also infect people leading in some cases to TB or possibly to Crohn’s disease which affects the human gut.

Bovine TB has been increasing in incidence in UK cattle herd for the past 30 years and current control measures lack sensitivity and specificity. Confounded by the presence of a wildlife reservoir of disease, future control of TB in cattle will likely depend on vaccination. However this requires significant improvements in our understanding of how TB vaccines, including the human TB vaccine BCG, work. This requires further studies of the immune

response. Currently, cattle are not vaccinated against TB, and the diagnostic tests are limitations.

Johne's disease is a significant cause of economic losses worldwide with significant impact on milk production and meat quality. As with bovine TB, current control measures lack sensitivity and specificity and no vaccines are currently available.

In order to control these diseases in cattle to significantly impact animal health, and the farming economy, we need to develop new vaccines or ways to identify infected animals so we can treat or remove them from farms. Developing new vaccines or diagnostic tests requires us to understand the immune response that can lead to immunity (versus disease) and also to understand why some animals respond differently to each other (the role of host genetics). This in turn will lead to improvements in human health through disease control.

Our previous work showed that some vaccines work better than others and we have started to identify parts of the immune response that help vaccines work well. We need to understand this in greater detail so that we can provide information that will, in the future, help to make better vaccines or tests for diseases and also perhaps to breed resistant animals.

Therefore, the major aim of this project is to understand how these Mycobacteria interact with components of the immune system and how this differs when cattle are vaccinated before being infected. This will help us define targets to work with in the future. We can use animals known to have different genetics to help determine whether we can breed for resistance. We will carry out a small number of experiments with other pathogens (parasites, bacteria, viruses) for comparison.

What outputs do you think you will see at the end of this project?

The major output from this project will be the generation of new information on the immune responses of cattle to vaccines and disease. This is essential to allow us to move forward with the development of new diagnostic tests, vaccines and other disease control methods. We will publish these outputs in scientific journals and we will interact with the public and our stakeholders (companies, farmers etc.) to share what we find.

Who or what will benefit from these outputs, and how?

By determining how immunity can be stimulated by vaccines, what cells and mechanisms are involved we will provide helpful information for a number of scientific studies looking at the development of new ways to control Mycobacterial diseases (of cattle but we will also influence studies of related human diseases). We expect within the next 5 years to use the information to begin to work with companies to provide information that will allow them to develop new vaccines or tests for disease; this could include the registration of at least one new product and a patent. These studies are ultimately aimed at producing effective vaccines, diagnostic tests and other control measures against diseases of livestock that have significant impacts on animal health and the economy. These will mostly occur within the next 5-10 years.

How will you look to maximise the outputs of this work?
We will collaborate widely across the veterinary research community to disseminate our findings: this will be through our existing networks, through dissemination activities at scientific conferences and by publication. We will contribute widely to outreach events

aimed at both the public and stakeholders. It is ever-important to publish unsuccessful approaches to minimise the likelihood of duplication of failed studies. Our scientific rigour includes the dissemination of 'negative' results for this purpose.

Species and numbers of animals expected to be used

- Cattle: 170

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project uses cattle as these are the natural hosts for the important diseases we wish to study. We will use both juveniles and adults. Juveniles at the age of approximately 8 weeks have been weaned and are relatively small in size and easy to handle for the studies of cells draining in the lymphatic vessels as these require surgical preparation. Calves at the age of 6-8 weeks are known to respond more effectively to some vaccines being used to control tuberculosis and understanding why they show strong protective immunity at this age is a major objective of our work. Older cattle including young adults will be used as blood donors, in vaccination and infection studies and will be also sampled at farms where they are of known natural infection status.

Typically, what will be done to an animal used in your project?

For typical investigations on cattle they will be subject to blood sampling to access the cells and proteins of the immune response. Some animals will receive vaccines, or other substances designed to induce protective immune responses which will be measured in the blood or in relevant tissues such as the skin. Some of these animals that have been vaccinated, or non-vaccinated controls, will be infected with the pathogen of interest. After vaccination and challenge samples will be collected on a regular basis to determine if the pathogen is present and if an immune response against the pathogen can be detected. At termination of the project the majority of animals will be killed using a Schedule 1 method, those which are not killed will be re-homed. Animals may be subject to post-mortem examination where the extent of disease in infected animals is assessed, and tissue responses assessed.

We will also examine the immune response of cells as they circulate from vaccination sites to the rest of the body: this involves surgical preparation and insertion of tubing to allow collection of cells. These animals recover from the surgery and we can collect these cells over a period of up to one month.
Some animals will receive vaccines and the response will be monitored over time. Animals may also have skin biopsies taken, or samples taken from the lung: this will be done under local anaesthesia or sedation. These animals will be killed at the end of the study using approved Schedule 1 methods.
Blood donor animals that are non-vaccinated or non-exposed, and naturally infected animals that are sampled at farms may be kept alive and released from the controls of the Project Licence.

What are the expected impacts and/or adverse effects for the animals during your project?

Blood sampling: temporary discomfort at the sampling site, a very small proportion (<1%) may experience haemorrhage but this is unexpected.

Lymphatic cannulation: short term seepage of fluid following lymph node removal for less than 48h. Anaesthesia may induce adverse reactions (<1%). Following surgery analgesia will be given.

Inoculation/vaccination: The administration of substances will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. There may be mild local inflammation at the injection site lasting no more than a few days.

Infection with Mycobacteria: using the doses and regimes we have refined in previous studies animals are not expected to show any clinical signs other than transient (less than 48h) alterations in temperature, respiratory effort or diarrhoea.

Tissue sampling from the skin or lung: mild discomfort may occur and where necessary analgesia will be given. Skin biopsies will be taken under local anaesthetic. Lung sampling will be done under sedation to minimise the likelihood of damage to the respiratory tract during endoscopy.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Cattle will undergo mild or moderate severity procedures. The vast majority (~80%) will be mild and approximately 20% moderate (surgically prepared calves for collection of samples from the lymph).

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

The objectives of the project cannot be achieved without the use of cattle. There are no good alternatives that can be used: mice can be infected with Mycobacteria but they do not have the disease outcomes as cattle and cell lines are not available to use in the lab. However, where we can we will use cells derived from blood and tissues to mimic responses that occur within animals. For example, we can use new technology to generate ‘mini-guts’ (known as organoids) in the lab to look at the early interactions of the bacteria

that Johne’s disease with the gut or we can use blood derived cells to understand how TB causing bacteria ‘hide’ from the immune response and cause infection/disease.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we will use alternatives but as detailed above many of these have animal origins; for example the use of organoids requires animal tissue. There are no viable non-animal alternatives for our studies.

Why were they not suitable?

Our alternatives all require a source of animal material. While cell lines and organoids can be used to address specific questions, understanding how the whole animal responds to a vaccine or to disease cannot be mimicked in the lab due to the complexity of the body systems involved.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 more than 20 year's expertise in the type of experiments being carried out and have used this, and a vast collection of data, to estimate the numbers we will use. We have consulted with experts in statistics to use the data and expertise to design our experiments optimally.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Good principles of experimental design are always used to ensure that we used the minimum number of animals to achieve robust and reliable results. All of our experiments are officially reviewed by a named vet, animal care technicians and a statistician before they can be undertaken. These study protocols include aims, numbers of animals, treatments, assessment of adverse effects, end points and our data analysis methods which are carefully scrutinised. We will refer to the NC3R's experimental design assistant as required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
Where we do not have extensive experience, or where there is limited information we will perform pilot studies to determine outputs that will inform experimental design.

In addition, we will archive samples to allow additional analyses to take place without the need to repeat experiments in animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use cattle in this project. These animals will be naive (non-vaccinated, non-diseased), vaccinated or infected with pathogens that cause disease. We will use disease models that have been designed to allow us to answer important questions with the minimum of pain, suffering and lasting harm. We will use doses of infectious agents and time-scales that do not cause overt disease or suffering and we will continually assess our methodology and refine where necessary. These parameters allow us to mimic the natural disease but with the minimum harm. The majority of animals will be blood sampled, with a smaller proportion undergoing surgery or infection studies. All animals undergoing infectious challenge or surgery will be monitored at least twice daily using well-defined clinical scoring criteria. This will allow us to continually monitor the wellbeing of the animals to minimise harm.

Why can’t you use animals that are less sentient?

Cattle are the natural hosts for the pathogenic organisms being studied and no accurate models are available. Vaccination and challenge studies will enable us to pinpoint critical factors associated with the induction of immunity, or the progression to infection. We can define both immune and genetic factors enabling well defined experiments to be carried out. These factors cannot easily be replicated in less sentient animals. The nature of our studies requires longitudinal analyses of animals as they respond to vaccines or infection; this could not be done in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have already refined a number of our protocols. For example, we have refined the post-operative care that calves receive to reduce stress to the animals from injection of anti-coagulants (these animals were previously observed have become needle-shy with 2x daily injections for up to 28 days; reducing this [to 2x daily for 3 days then daily for up to ~10 days] has had a positive impact on these animals). We will administer analgesia as required to animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to guidelines published by the UK National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will monitor correspondence and guidelines from the NC3Rs on a regular basis and implement relevant changes in a timely manner. The project licence holder is in frequent

contact with the named veterinary surgeon and the named animal care workers: this will enable any changes to be made where appropriate.

165. Development and function of the mammalian brain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

neuronal progenitors and stem cells, neurogenesis, neuronal migration, formation of neuronal connections, brain developmental abnormalities

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to study the basic steps of brain development (neurogenesis, neuronal migration, development of connectivity) to understand the molecular and cellular mechanisms determining functional cortical circuits with particular attention to the early circuits in the cerebral cortex. We also study the effects of silencing or stimulating a selected neuronal population either during development or in the adult and examining the effects on development and maintenance of connections, myelination, microglia activity and behavioural changes of the animals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The causes and remedies of many cerebral cortical developmental disorders are not known, but their prevalence in the general population numbers is high [schizophrenia (1:100); autism (1:68); attention deficit hyperactivity disorder (1:30); dyslexia (1:10); childhood epilepsy (1:200); neural tube closure defects; cerebral palsy (2:1000)]. Without the understanding of the basic developmental mechanisms such as how neurons are generated, how they migrate, differentiate, develop connections, and form functional assemblies it is impossible to contemplate that we can develop interventions to prevent or treat these conditions. It is not enough just to study the adult brain, since the developing brain is not just a smaller version of the adult brain. The management of developmental conditions requires very different approaches than in the adult. The specific and selective vulnerability of the developing brain to hypoxia, ischemia, maternal infection is still not understood, but the impact on the life on the individual and their family can be devastating. These particular studies could have direct implications on establishing general policies e.g. vaccination during pregnancy, with or without particular genetic susceptibility or management of neonatal hypoxia-ischaemia or the management of genetic or acquired conditions.

What outputs do you think you will see at the end of this project?

We shall reveal basic cellular and molecular mechanisms that are involved in the normal and abnormal development of the mammalian brain, with special attention to the cerebral cortex. We shall understand the background of some basic developmental abnormalities (such as alterations in neurogenesis, neuronal migration, formation of connections) that underlie some devastating conditions and manifest as childhood epilepsy, learning difficulties, cerebral palsy. These observations will be published in the scientific literature and some of these mechanisms will be validated in human brains.

Who or what will benefit from these outputs, and how?

Some of the outputs of our PPL will continue to contribute to our long-term goals to bring greater understanding of these basic neurobiological problems. We shall identify specific cellular and molecular mechanisms that are responsible for developmental steps. This better understanding will elevate the platform from which the prevention and treatment of many neurological and psychiatric disorders (e.g. childhood epilepsy, schizophrenia, attention deficit hyperactivity disorder, autism) can be contemplated. During the licence we shall identify some mechanisms that will eventually bring novel therapies and preventions to these conditions and disorders that affect millions of people of all ages at tremendous cost to the national economy.

Understanding the possible dangers during brain development can help with prevention or treatments of several neuronal developmental disorders. We hope to contribute to the basic knowledge base that, in time, shall have a very significant impact on this field, including clinical diagnosis and possible treatment.

Our work will provide novel knowledge that will improve reproducibility of rodent studies and will help to make the mouse a better model for biomedical research.

We expect to achieve some of our outputs in the life of this PPL, such as molecular and cellular susceptibilities of hypoxia-ischaemia, neural plasticity, role of cell populations in brain state control, but these outputs that will be published in the scientific literature and presented to colleagues and clinicians will be only the first steps towards clinical translation.

How will you look to maximise the outputs of this work?

Our laboratory is performing fundamental basic research, primarily to improve our understanding of the developing brain. We shall make our research results accessible in an open format to all our collaborators, and to all researchers through our detailed publications. To disseminate knowledge, increase impact and encourage reproducible science we deposit the preprints of our publications with open access even before our papers are accepted for publication. Members of our laboratories regularly present their data on national and international meetings and workshops. Members of the laboratory are also involved in the training and teaching of the next generation of biomedical and medical experts and through this teaching disseminate new knowledge. We regularly publish both positive and negative results and share successful and unsuccessful approaches with our colleagues.

Species and numbers of animals expected to be used

- Mice: 24,000
- Rats: 2,980

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We study the development of the cerebral cortex in mouse and rat. Rats and mice are the lowest vertebrate group that the above-described experiments can be performed in. The presence of isocortex (sixed-layered neocortex) is limited to mammals; therefore, the use of fruit fly and zebra fish is limited to certain specific questions relating to gene interactions and cascades. The genetic manipulations and the transgenic and mutant mice models contribute a great deal to the understanding of the cerebral cortical development because of the similarities of the basic patterns of cortical development to primates.

Although mice will be used in most cases, the use of rats in certain experiments is apparent. Rats could be more practical for some intrauterine surgeries or central nervous system lesions or for implantation for slowly releasing polymers purely because of their larger size. Also, rats could be more suited for some immunohistochemical analysis because numerous antibodies are produced in mouse. There is also growing evidence of differences in the cell-surface receptor expression in cerebral cortical neurons between mouse and rats, which may result in different cellular or whole animal responses for state control experiments. However, the reporter gene expressing transgenic mouse lines and mouse mutants make this species the preferred choice for most of our experiments. Also, implantation of tissue from other species (e.g. 3D printed constructs from human induced pluripotent stem cells) require immunodeficient mouse hosts. These hosts accept grafted constructs where their long-term interaction with the host can be studied in detail.
For our experiments we shall use several life stages, including embryonic, postnatal, and adult stages. We use embryonic stages to study the process of neurogenesis, neuronal migration, and formation of early connections. Neonatal stages provide access to study early circuit formation and plasticity. Adult stages will be used to study normal and abnormal organisation after normal or disturbed development. The alterations in development will be also studied in adults.

Typically, what will be done to an animal used in your project?

Most animals used in our projects will be used for histological and molecular studies without surgical procedures after collecting their brains after perfusion in terminal anaesthesia at various life stages. However, in some experiments the pregnant dams will need to undergo laparotomy to allow to perform manipulations, labelling, lesioning specific cell groups or structures. Some animals will have postnatal or adult manipulations to monitor or to modulate the function of particular cell populations or structures. Some of these animals will have genetic modifications to reveal specific neuronal populations with the expression of reporter genes and some will have genetic modifications through specific breeding protocols. The animals will be bred, and several developmental stages examined after terminal anaesthesia. Only a small fraction of the animals will go through early manipulations either in utero or at perinatal stages. Most of these animals will go through a single manipulation, but occasionally there will be some animals with multiple procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically altered animals bred in this project are not expected to show any adverse effects before they are weaned. Some of our models will show some mild behavioural changes normally after 6 months of age.

A large part of our study involves the manipulation of the embryonic and adult brain. Cell labelling drugs are not expected to have any effect, but substances to cause gene alterations administered in the pregnant dam can cause transient weight loss. Physical intervention to the embryonic brain such as injection of constructs or expression of constructs with electroporation will require the pregnant dam to undergo surgery with a risk of the embryos being aborted. Animals born from these procedures may exceptionally show some subtle behavioural changes such as altered sensory perception or fine movement alteration (e.g. layer 5 alterations can change grasping), but we observed none of these over the last 5 years.

In addition to the initial manipulation in utero animals may undergo further manipulations as an adult.

Animals may undergo a selected group of behavioural tests to enable us to study the effects of the brain manipulation. These tests are not expected to cause any additional harms to the animals.

Some of the animals that undergo hypoxia-ischemia are expected to show weight loss, lethargy, poor motor control, and seizures. Our research will use paradigms that are mild or moderate to determine the factors that help in the recovery. We shall study the alterations in movement and memory and shall manage the long-term effects.
Where animals need to undergo surgery for manipulations it is expected that there will be transient pain from wounds. Analgesics will be administered during and up to 2 days post procedure until the animals are sufficiently recovered from surgery.

We will harvest embryos to explore if development can be delayed or altered under laboratory conditions.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse 70% mild, 30% moderate,

Rats 70% mild, 30% moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Rats and mice are the lowest vertebrate group in which our experiments can be performed. Our studies are centred around the isocortex in the brain which is only found in mammals therefore the use of the fruit fly or zebra fish is limited to answering specific questions relating to gene interactions and cascades. Genetically altered rats and mice contribute to the understanding of the cerebral cortex development because of the ability to create basic cortex development patterns seen in primates without the need to use higher species.

There is no alternative model. The questions cannot be answered in human.

Which non-animal alternatives did you consider for use in this project?

Computer model

Mathematical modelling

Human induced pluripotent stem cell - 3D tissue printing

Human organoids

Study postmortem fixed pathological material.

Why were they not suitable?

No computer model is currently available that can replace the use of animal tissue for our objectives, as there is insufficient information on the network connectivity and circuit activity involved. Nevertheless, in the future computer models may be used to assist the interpretation of the data obtained in experiments from animal tissue.

Our laboratory is increasingly using mathematical modelling to identify the key factors involved in cerebral cortical neurogenesis and to identify the most sensitive parameters to study in biological systems.

We currently working on technologies to 3D print cortical layered constructs from human induced pluripotent stem cells, but this methodology is still at its infancy. Our collaborative team is one of the first to attempt this. These models can only recapitulate the earliest stages of cerebral cortical development and they do not reach the level of sophistication as the real tissue that developed in vivo. To explore more complex in vivo interactions, we implant these 3D printed constructs into mouse brains.

Human organoids only model the very early stages of cortical development and they do not possess the complex circuits, such as the thalamocortical connections.

We study human post-mortem fixed pathological material, but these only allow descriptive 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 number of animals estimated to be used in this project comes from experience gained in managing experiments of the type proposed in this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have investigated possible means of reducing the number of animals that we will use by exploring NC3R's Experimental Design Assistant (www.nc3rs.org.uk/experimental-design-assistant-eda). This online tool helps us through the design of our experiments ensuring that we use the minimum number of animals consistent with our scientific objectives, selected methods to reduce subjective bias, and appropriate statistical analysis.

We can minimise the use of animals by considering the influence of specific variables and addressing sources of possible bias. We developed experience from our previous published studies how to design our specific experiments that will produce robust and reproducible data.

We used mathematical modelling to identify the most sensitive parameters that should be studied in detail (e.g. in cortical neurogenesis). This helped us to identify factors that influence our experimental results the most. NC3R's Experimental Design Assistant also advised us the use of the most efficient statistics. This can also reduce the number of animals required and maximise the information obtained per experiment.

To further increase the reproducibility and validity of our experimental research we use PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) – (https://norecopa.no/prepare). This resource gives valuable guidance for formulating of the study, good communication between researchers and biomedical facility and methods.

We will ensure the minimum number of animals used whilst still enough for statistical analysis. For this, experimental design will be optimised to obtain answers to the questions addressed, and statistical power analysis will be employed ahead of commencement of experiments. We shall be conducting all our experiments so that we comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines) when we publish. This will enable the scientific community of readers and reviewers to scrutinise and reproduce our research.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Good colony management will ensure that we only produce those animals required for our experiments and to maintain the colony. Where the genetical manipulation allows we will breed homozygous animals to further reduce the number of animals being produced. Details concerning strain data will be obtained from the inhouse animal tracking database. Where possible we will share our transgenic rodent colonies with other groups within our establishment to reduce the need for duplicated colonies.

We shall optimise the number of animals used by relying on our considerable pilot studies. Most of the proposed experimental methods have been used in our laboratory previously and this helps with the estimation of the number of animals to reach our objectives.

We have already used computer modelling is selected suitable areas to explore the most important parameters of cerebral cortical neurogenesis to guide our studies. We shall continue to collaborate and consult with our mathematical modelling colleagues to explore and identify the most biologically relevant mechanisms that we should explore with experiments in more details. These decisions will optimise the use of animals.

We archive our experimental material, so the raw data is available online to the entire laboratory. This can reduce the use of new experimental animals on questions that can be analysed on archived material. We also established several web-based resources to share results on gene expression patterns to reduce animal use of new animals. Our laboratory pioneered the establishment of transcriptomic atlases and posted these freely to the broad scientific community.

By harvesting and storing as many post-mortem tissues as possible from our animals we can reduce the need for new experimental animals to answer some of our questions from this archived material. We will also offer other post-mortem tissues that are not relevant to our scientific question to other groups within the establishment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of species and models:

We will use rats and mice for this project. We carefully considered the methods chosen for our objectives and made sure that they will cause least harm, whilst achieving our scientific objectives. Rats and mice are sufficiently close to humans to reveal principles of brain development and are species that are much used in this area, which enables us to build upon a large body of research already carried out, and to relate our findings to previous results. Whilst the bulk of experiments will be done in normal animals, we wish to utilise several reporter and knockout models to focus the mechanism of a particular gene in cortical development. At present our genetically modified mice do not express any severe phenotypes, but any additional GM mice imported into this licence would be carefully monitored and ensure any adverse phenotypes do not exceed the severity limit. The rat line (GAERS - Genetic Absence Epilepsy Rat from Strasbourg) is a recognized animal model of absence epilepsy, a typical childhood form of epilepsy. It is spontaneous mutant on the Wistar background. The strain will help answer questions in relation to absence epilepsy in the developing and adult brain.

Choice of methods:

We will use methods that will cause the least pain, suffering, distress, and lasting harm. Our main methods include the labelling of neurons and their projections of gene expression with histological methods that do not require living animals. We shall use methods when the genetically modified animals will be terminally anesthetised and perfused with fixative to preserve the structure of the brain tissue for further investigations with post mortem tracing methods (using lipid soluble tracers that travel in fixed tissue of developing brains), study gene expression with immunohistochemistry, in situ hybridisation. Some of the tissue will be collected in similar manner to keep the brain tissue alive in isolation for stimulation and recording ex vivo. This is the most refined model that can be used for the study of these questions. We shall only use in vivo methodologies when it is necessary for conclusive experiments. We shall use chemical (gene deleting/induction agents) or physical (electrophysiology) and at various stages of development (embryonic, postnatal and adult). We will always follow best practice and asepsis at all times.
We shall use manipulations that are performed at early developmental stages (in utero or immediately after birth) to alter the development of the pups. With the intrauterine manipulations we deliver constructs into specific cohorts of newly born neurons from the lateral ventricles to trace, up-regulate or down-regulate a specific gene function. For some experiments we shall use physical means of creating lesions such as whisker removal or whisker trimming or disruption to the infraorbital or optic nerve or the eye itself. These methods are essential to study the long-term systems effect which is not possible in in vitro culture system. Although it is not a complete replacement, it is increasing used as an alternative than creating knockout or knock in genetically modified animals.

These are considered the least severe procedures to answer the scientific questions related to the role of sensory input in brain development. We shall only remove one of the eyes (instead of both) to reduce the lasting effects on the animas. There are other genetically altered mice that could be used in these experiments (develop smaller or no eye) but they exhibit other developmental harmful phenotypes which if used would increase the level of harm to the animals.

We model developmental conditions that can have devastating effects on human infants, such as maternal inflammation and hypoxia – ischaemia. We will place animals in altered

conditions to mimic the effects seen in the human such as maternal inflammation and hypoxia-ischemia with or without ligation of some blood vessels supplying the brain. Animals will be placed into conditions where there are altered gas levels (hypoxic conditions for 1-2 hours) to produce mild or moderate hypoxia to keep suffering to a minimum. It is not expected that any deaths will result from the hypoxia as the protocol is well established, however there is the risk of maternal rejection on returning the litter to the dam.

The corresponding animal models will show some adverse effects (e.g. maternal inflammation induced by toxic molecules from outer membrane of Gram-negative bacteria, such as lipopolysaccharides; maternal stress caused by amphetamine intake), but these effects cannot be ameliorated because we specifically would like to study these maternal effects on cortical development in foetuses. However, we will not use doses that cause high abortion rates or severe phenotypes in dams, as that would render the animal samples collected not fit for the purpose of the experiments. Similarly, adverse effects are expected in pups subjected to the perinatal hypoxia-ischaemia protocol and in some adult animals showing neurodegeneration following gene manipulation in selected cell populations. In both cases, the manipulation is necessary to investigate the proposed scientific questions. Suffering will be kept to a minimum by determining the shortest survival post-onset-of-symptoms necessary to address the scientific questions.

We will use the least aversive behavioural tests that are well established to enable us to assess the effect of our manipulations on the animal’s ability to perform such tests.

Whenever possible we will harvest brain tissue under terminal anaesthesia and use post-mortem tracing with carbocyanine dyes, a non-invasive method that we pioneered.

Why can’t you use animals that are less sentient?

We study cerebral cortical development and some of our experiments are at very immature life stages when most of the neurons are generated and they still in migration to their destination. We also have to study later stages to explore the consequences of altered development.

Our work revolves around the development of the mammalian cerebral cortex which is not present in a lower species such as the fruit fly or zebrafish. Species such as birds and amphibia do not have the complex mammalian brain required for our work.

For most of our work we use animals that have been terminally anaesthetised, and tissue has been collected for future molecular or histological analysis. Some of the tissue kept alive for in vitro experiments that allow some degree of manipulations. We can perform some brain manipulation whilst the animal is terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We shall use good aseptic practice when performing surgery. All stereotaxic injections into the brain, intrauterine surgery, and peripheral or central nervous system lesions will be done under aseptic conditions to prevent any post-surgery infections under general anaesthesia

We shall use increased monitoring during and after surgery to observe the condition of the animals and the level of anaesthesia. We use postoperative assessment charts to keep record of the recovery.

The most appropriate inhalation anaesthesia will be used during surgeries.

We shall manage pain during and after surgery. Analgesics suitable for the species will be applied peri- operatively as standard to minimise suffering and monitored regularly for at least three days (at least once daily in addition to the normal checks by the technicians). A general pain-scoring chart will be used to record the well-being of the animals, as well as any adverse effects specific to the operations performed on that animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow LASA guidelines on best practice (https://www.lasa.co.uk) to improve animal welfare and quality of science.

We follow the NC3R's Experimental Design Assistant (www.nc3rs.org.uk/experimental-design- assistant-eda) to make sure that the experiments are conducted in the most refined manner.

To further increase the reproducibility and validity of our experimental research we use PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) – (https://norecopa.no/prepare).

We shall be conducting all our experiments so that we comply with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines) when we publish. This will enable the scientific community of readers and reviewers to scrutinise and reproduce our research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay informed about advances in the 3Rs by regularly attend the welfare meetings and 3Rs Research Day at our establishment.

We subscribe to the NC3R's website (https://www.nc3rs.org.uk) to get information on 3Rs resources, news, and events.

We have regular interactions with the NC3Rs Regional Programme Manager who also advise us on available grant support and 3Rs studentships. Our laboratory submitted application for both grants and studentships in the past.

Our laboratory currently collaborates with the Chair of the 3Rs subcommittee and learn about the best practices through joined laboratory meetings and discussions.

All new members of the laboratory will receive specific 3Rs training that will focus on the development and use of alternative platforms to study human cerebral cortex development. The specific 3Rs training will include experimental design (including the use of the NC3Rs EDA), implementation of the ARRIVE guidelines (especially randomisation/blinding), development of non-invasive imaging methods, development of other non-invasive assays for studying physiology and behaviour in mice and aseptic stereotaxic surgery. Furthermore, high animal welfare standards are essential in all

research. Key element of 3Rs specific training in this project will be the importance of good animal welfare for high quality scientific data.

166. Molecular mechanisms regulating sleep and 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Sleep, Circadian rhythms, Neuroscience, Signalling

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the molecular mechanisms that generate and regulate sleep and circadian rhythms.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Sleep and circadian rhythms (SCR) are a core component of our physiology, and their disruption (SCRD) leads to several acute and long term health consequences including mental health conditions, metabolic syndromes, and neurodegenerative disease.

Furthermore, SCRD is a feature of most neuropsychiatric conditions and core determinant of quality of life, but is rarely treated. This is mainly as we understand very little about the molecular pathways that control sleep and circadian rhythms. This project aims to develop the substrate with which novel therapeutic interventions that target these pathways can be developed

What outputs do you think you will see at the end of this project?

An increased understanding of the mechanisms by which SCR are regulated leading to

publications

novel targets for drug development

patents on translatable findings

novel small molecule or biological drugs for the treatment of SCRD that will be developed in collaboration with pharmaceutical companies.

Who or what will benefit from these outputs, and how?

The publications and information on novel targets will be of benefit to the scientific community and other stakeholders including policy makers, advisory bodies and the pharmaceutical sectors. The outputs will be seen within 5 years.

Patents will ensure proper commercialisation of the findings from this research. Preclinical development of novel drugs will facilitate the development pipeline of novel therapeutics. For example, we will identify the best in class compounds, with the appropriate readouts of efficacy, dosing information and route of administration to enable transition to clinical trial. These benefits will be realised within 5 years.

How will you look to maximise the outputs of this work?

Dissemination of new knowledge: Outputs will be presented at conferences and as online preprints prior to publication in peer reviewed journals. All our work will be open-access and published with access to full raw data. Our work generated large datasets of interest to the entire neuroscience community, and these will be freely distributed.

Commercialisation: Patents will be filed through relevant channels and licensed, leading to spin-out companies or collaborations with the biotech/pharma sectors.

Species and numbers of animals expected to be used

- Mice: 8500
- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Mice are the species of lowest neurophysiological sensitivity that are appropriate for such studies, and have well-characterised circadian and sleep biology. Extensive genomic data coupled with the wide range of existing genetically altered models make mice the only viable model for such studies. Adult animals are required for behavioural monitoring. Aged animals (over 15 months, but less than 2 years) will be used to model the sleep and circadian disruption seen in the elderly. Mice will also bred through to the adult stage under conditions of circadian disruption to model the effect of shift-work/sleep disruption on reproduction/epigenetic inheritance.

Adult rats will be used for experiments on circadian rhythm entrainment for a limited number of studies where mice are unsuitable, as this parameter is very sensitive to slight environmental changes in mice.

Typically, what will be done to an animal used in your project?

Animals (either wild type, or genetically altered to target specific genes in sleep/circadian rhythms) will be housed in a cage equipped with a running wheel/other equipment to monitor behaviour in a chamber in which the light environment can be controlled, to be able to simulate an environment that disrupts sleep and circadian rhythms (similar to a shift work environment). The animals will receive an intervention, which is typically an injection of a compound being tested, and their sleep and circadian behaviour in response monitored. Most animals will be monitored for 4-6 weeks, with <25% of experiments lasting 3-6 months. Occasionally, samples such as blood may be taken from the animals, or an animal may undergo surgery to allow for the implantation of electrodes to enable recording of sleep EEG, or administer drugs/biological substances directly into the brain, if they cannot enter it from the bloodstream.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will have deficits in sleep and circadian rhythms resulting from modification of the environmental light/dark cycle, genetic alteration or the administration of drugs. Any adverse effects are typically mild (distress from single housing, temporarily heightened or reduced activity, some strains may show weight loss or gain) and mice will not be kept in an experiment beyond 12 months and not maintained beyond 15 months, with an exception made for experiments with aged mice (<5% of the experiments), which model the significant sleep/circadian rhythm disruption also seen in aged humans.

The animals may undergo surgery to administer substances intracranially or via implantation of an osmotic minipump, or to implant electrodes, and will experience transient discomfort associated with surgery (< 1 week), mitigated with analgesia. Most of the substances administered will be well characterised, and few side effects expected.

The models required for studies on metabolism may be diabetic and/or obese, typically induced by feeding of a high fat diet for 3-12 months or by a genetic alteration in the satiety pathway.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice 50% Subthreshold, 25% Mild 25% Moderate

Rats 100% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This work involves the study of complex physiological and behavioural systems. This necessitates the use of whole animals as no suitable alternatives exist. Mice are the species of lowest neurophysiological sensitivity that are appropriate for such studies, and have well-characterised circadian and sleep biology. Extensive genomic data coupled with the wide range of existing genetically altered models make mice the only viable model for such studies.

Which non-animal alternatives did you consider for use in this project?

We have investigated the use of 1) Cultured cells 2) *ex vivo* tissue 3) human stem cell derived neuronal cultures/fibroblast cultures for our studies. All of these are currently already used in our group as models in which to investigate the molecular basis of the genes/drugs we investigate.

Why were they not suitable?

None of the models listed above can fully recapitulate in vivo sleep and circadian rhythms, which are a behavioural readout resulting from complex organ/system level interactions. Whilst the majority of mechanistic analysis will be carried out in the models above, final validation of the resulting changes in sleep/circadian behaviour will require animal experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

Numbers for is this project have been estimated from previous experience of experiments of this nature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Efficient planning to make sure maximum data output from a single experiment; for example collection of tissues for omics analysis at the end of a protocol.

Validation of which aspects of the investigations can be replicated in vitro; for example circadian period can be accurately modelled in vitro requiring far fewer animals for in vivo experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Target identification from in vitro high-throughput screens and large datasets, allowing multiple targets to be investigated in parallel.

Validation of target action in vitro, with only significant results being progressed to animal studies.

Efficient breeding with use of littermate controls and both sexes wherever possible. Sharing of tissue and efficient archiving of samples from each experiment.

Pilot studies with small cohorts will be conducted where appropriate to determine statistical power.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use primarily mice, as a large amount of genetic information and genetically altered models suitable for circadian rhythm and sleep research are available. Some assays will require rats, where the mouse's sensitivity to particular stimuli, such as handling, that control circadian rhythms makes it unsuitable as a model, but rats will be limited to less than 5% of the overall experiments.

The methods in majority have been optimised to cause only mild and transient distress. These include alteration of the light dark cycle (for example to simulate jet-lag), monitoring of activity using non- invasive methods, the occasional withdrawal of blood from a superficial vessel and the occasional administration of drugs / other substances and the alteration of diet. In some cases, these methods cumulatively cause moderate distress to be experienced, for example daily dosing of a drug by injection, but these methods will be optimised to cause the lowest experimental load to be experienced by any animal.

Why can’t you use animals that are less sentient?

Mice have the lowest neurophysiological complexity to still be useful for the research outlined, which requires a complex nervous system. Circadian rhythms and sleep are behaviours that need to be assessed in freely moving adult animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In most cases, the animal's activity will be continuously monitored using an online readout, with automated alerts to the experimenter in case of any gross alterations. Where activity is not monitored, the animals will be monitored as frequently as appropriate for the intervention; for example twice- weekly weighing when a new drug/diet is administered until a stable baseline or trajectory is reached. Animals will also be habituated to any handling of changes in the environment prior to experiments (e.g. gentle handling, exposure to novel environments). Peri-operative care and pain management will be provided as standard.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will reference the below websites, in addition to following the ARRIVE guidelines, a 20-item checklist designed to improve the reporting of research using animals widely implemented in many journals. We will also follow literature specific to the field of circadian rhythms that specify experimental design protocols to ensure reproducibility with minimal animal numbers and suffering (e.g. in the journal of biological rhythms).

www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Subscribe to the 3Rs newsletter and attend the termly Animal Welfare meetings organised within the institution, and also regularly attend the 3Rs conference. The following websites will be referenced: www.nc3rs.org.uk, https://science.rspca.org.uk.

We will try new advances including non-animal based models of circadian rhythms for their capacity to replace some of our animal experiments and already use a wide variety of such technologies.

167. Understanding the role of the host immune response and tumour microenvironment to enable the generation of novel immunotherapeutic strategies to treat cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, antibody, immune-modulation, microenvironment, host factors

Animal types	Life stages
Mice	adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to understand the role of the host immune response and tumour microenvironment in cancer to enable the generation of novel immunotherapeutic strategies for clinical application. The specific objectives are:

Determine the mechanism of action of monoclonal antibodies (mAb), how they may be potentiated by pharmacological drugs and/or immune-modulatory agents/and or other modalities with the aim of developing improved clinical reagents;

Understand how the immune environment both systemically and locally changes and develops with cancer, how this is impacted by host factors (e.g. body composition) and the potential impact that this may have on anti-cancer immunotherapy;

Elucidate the mechanistic requirements for and then develop strategies to overcome cancer and/or host induced immune suppression thereby promoting immune responses to cancer using mAb, mAb derivatives and/or other immune-modulatory agents and delivery 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?

Cancer is the cause of one in four of all deaths in the UK with a greater proportion still affected by cancer during their lifetime. Despite advances in screening and diagnosis cancer incidence continues to rise, largely as a result of an aging population and the impact of our modern lifestyle. The majority of cancers are diagnosed late and treatment frequently involves surgery, accompanied by chemotherapy or radiotherapy. Despite these invasive and toxic interventions patients often relapse due to the survival of small numbers of tumour cells. In spite of decades of work on treatment regimens, the survival for many cancers remained unchanged until very recently. Immunotherapy, whereby the power of the patient's own immune system is harnessed to eradicate cancer cells, is an attractive adjunct to current treatments as it offers the possibility to be administered with maximal specificity, minimal toxicity and provide long term immune protection against recurrence. Indeed, immunotherapy has transformed the outcome of some cancer patients but these responses are variable and frequently limited to a minority of patients in a restricted number of malignancies. Clearly there is a need to develop new therapies for use either as an alternative to, or in combination with, conventional treatments.

What outputs do you think you will see at the end of this project?

Immunotherapy offers the potential to be applied to a wide range of cancers with highly specific reagents allowing us to treat patients by harnessing or effectively 'educating' the patient's own immune system to destroy unwanted cells while leaving normal tissue mostly untouched. Consequently, if applied appropriately, unlike conventional treatment, immunotherapies should not be associated with long-lasting toxicity. In addition, immunotherapy has the potential to provide vaccination leading to the establishment of immunological memory such that the patient's immune system will continue to recognise and kill their tumour cells on an ongoing basis, reducing the potential for relapse of their disease. Monoclonal antibodies (mAb) are the most successful immunotherapies translated to the clinic to date. Despite their undoubted impact, patient responses are variable and frequently limited to a minority of patients in a restricted number of cancers. Understanding what prevents more patients responding is a critical focus for much of the immuno-oncology research community and the focus of this programme. Through this programme we aim to understand how and why mAb immunotherapies work or fail in vivo (in live animals) and the role of microenvironmental and host factors in these variable responses. Through this better understanding we aim to deliver benefits through the development of new immunotherapy drugs and strategies that can then be tested clinically to enhance and widen patient responses. Other benefits are expected to emerge from the informed design of combination treatments in which mAb are given alongside conventional drugs and other emerging immunomodulatory (immune system altering) approaches. The ultimate aim is to develop and /or improve treatment options for patients with a range of cancers This will be achieved through the development of new treatment and/or reagents based upon a better understanding of the mechanisms involved. This may involve the patenting of new reagents/approaches as appropriate in collaboration with our funders and/or pharmaceutical companies. Several reagents developed under current and previous licences have been, or are already in, clinical trials. We have ongoing collaborations with at least six pharmaceutical companies and institutes for the development of new reagents and planned clinical trials.

We will also publish in peer-reviewed journals, present at scientific conferences and participate in public outreach activities. Primarily, our data will be of interest to scientists, pharmaceutical companies and clinicians. Principles established during these studies should also be applicable to immunotherapy against infectious diseases for both clinical and veterinary applications.

Who or what will benefit from these outputs, and how?

There will be multiple beneficiaries from our studies. In the long-term we aim for our primary beneficiaries to be cancer patients who we hope will benefit from our discoveries in the form of new and more effective treatments for their disease. In the short-term, we will publish our results in peer- reviewed journals, present at scientific conferences and participate in public outreach. Our data will be of interest to scientists, pharmaceutical companies and clinicians - and will serve to help them make further progress towards a deeper understanding of the complexity of immunotherapy in the context of cancer, its microenvironment and host. This greater understanding is anticipated to lead to new and better cancer drugs in the future. The benefits from our findings will not be entirely restricted to cancer as our aim in this context has strong parallels to the requirements for the immune system to fight infection. Therefore, the principles established during these studies should also be applicable to immunotherapy against infectious diseases for both clinical and veterinary applications. Furthermore, cancer and autoimmunity can be considered to be somewhat like opposite sides of the same immunological coin; in that the opposite effect to that required in cancer (where we seek to turn on an immune response where one is lacking) is required in the treatment of autoimmune disorders (where we seek to turn off an overactive immune response). This means that the drugs that we develop, should they prove to be inhibitors rather than activators, or the understanding that we obtain through our studies that may not be of use in the treatment of cancer may well be of use for the treatment of autoimmune diseases.

How will you look to maximise the outputs of this work?

We will aim to maximise our outputs by publishing and presenting our findings widely and freely, including in our University online open-access repository. Where possible we publish in open access journals, with full disclosure of the associated raw data, enabling our studies and results to be accessible to all. We have an excellent track record in publishing our findings (>60 to date with myself as an author), including those where approaches were not ultimately successful. In addition, almost all of our work is performed in collaboration with other academic groups and/or Biotech/Industry partners and so shared knowledge and expertise further maximises our outputs.

Species and numbers of animals expected to be used

- Mice: 14030

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Whilst all attempts are made to reduce the use of animals by using in vitro methodology (in vitro experiments on human and mouse immune cells, explant studies (tissue grown in culture medium out of the body) and organotypic models), it is inevitable in work of this nature that in vivo investigation must be undertaken. The animal models detailed are critical to facilitate in vivo proof of concept and efficacy in an amenable and manipulable system, not possible in humans. We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro and so systemic in vivo studies remain fundamental to the study of complex tumour microenvironments, host factors and therapeutics. Further, advances, such as the availability of genetically altered mice and the development of more specific and sensitive techniques and reagents, continually permit a refinement and reduction in the types of experiments and the numbers of animals that are required (e.g. as highlighted by the organisation 'Fund for the Replacement of Animals in Medical Experiments', http://www.frame.org.uk) and this is something to which we remain committed. The increasing availability of genetically altered mice has allowed genetic dissection and more meaningful modelling of human disease.

Adult mice will be used for this study since they have a mature immune system and are the most appropriate species of mammal that have the following characteristics:

The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

Individual mice within a given inbred strain are considered genetically 'identical', thereby reducing variability and allowing valid conclusions to be drawn from experimental data;

Numerous tumour models have been established in mice that are strain specific;

Numerous strains of genetically altered mice have been developed (often replacing the mouse receptor with the human receptor) several of which will be central to this programme of work and have been developed by us to answer our specific aims and objectives. These mice will allow us to understand the mechanistic requirements for effective therapy in 'human' relevant in vivo contexts. These experiments are simply not possible in humans and will enable us to validate and extend our findings in a 'human' setting and to help inform the generation of novel agents for use in patients.

Typically, what will be done to an animal used in your project?

This programme of work has 13 protocols that enable experiments to be conducted that meet our aims and objectives. The experiences of animals under these protocols will differ and below are detailed typical experiences for each:

Terminal bleeding for serum or cells. Mice (which may have been given special diet to alter their body composition) will be administered a suitable non-recovery anaesthetic and bled by cardiac puncture to obtain blood for serum or cells.

Tissue sampling and harvesting in the absence or presence of tumours. In experiments designed principally to measure effects on the immune system, mice (which may have been given special diet to alter their body composition) will be injected with material (such as an antibody or a combination of immunotherapy/immune-modulatory substances) and then the immune response measured by taking peripheral blood (much as we do for

patients) and measuring changes in the immune cells (typically over a period of days to weeks). These experiments are often done in the presence of a growing tumour, typically introduced earlier through injection (although mice that generate spontaneous tumour models may also be used), allowing us to assess infiltration of immune cells into the tumour after different treatments. Mice will then be euthanised and their tissues examined to understand how the immune response is developing in the different organs.

Immunotherapy of tumour models. In experiments designed principally to measure anti-tumour efficacy, mice (which may have been given special diet to alter their body composition) will be injected with an appropriate tumour model or mice that generate spontaneous tumours may be used and subsequently these would be administered treatments (such as a direct targeting and/or immune modulatory antibodies and/or other immune-modulatory substances) and tumour growth monitored over time through established means in experiments that may last several months, allowing us to determine if the treatments have cured the mice or delayed the tumour growth. Cured mice may be rechallenged with tumour or other sources of antigen to assess the induction of long-term immunological memory. A proportion of these mice may be monitored as above for their immune response during these experiments (peripheral blood or tissues).

Maintaining tumour cell lines and establishing humane endpoints. A minority of passaged (maintained) tumour models cannot be passaged in vitro whilst maintaining suitable characteristics for in vivo experimentation and therefore these models need to be passaged in vivo. Typically, mice will be administered a defined number of tumour cells and the tumours monitored and permitted to grow to the minimum point required to permit subsequent use of this tumour material for appropriately powered experiments. We constantly strive to use the most appropriate model to address our scientific objectives and therefore periodically need to acquire or develop new models which need to have their humane tumour endpoints established to ensure we can reproducibly use the models for our objectives whilst minimising welfare impact. Typically, a small number of mice (which may have been given special diet to alter their body composition) would be administered tumour cells by an appropriate route and tumour growth and animal welfare monitored to determine a suitable endpoint and ensure no unexpected or excessive harms are evident. These would be followed by pilot tissue sampling and harvesting or immunotherapy experiments to ensure the utility of the model.

What are the expected impacts and/or adverse effects for the animals during your project?

We typically identify mice by ear notching - this is expected to result in only mild and transient pain with no healing problems. Some mice in our studies are provided ad libitum altered diet (e.g. high-fat diet) to alter their body composition, or agents in their water to induce gene expression. Although these have the potential to impact welfare we have not observed these in our studies to date.

Mice will be injected with various immune-modulatory substances using a combination of volumes, routes and frequencies. Injections will cause momentary needle stick pain, minimised by using the needle of the smallest suitable gauge. The minimum number of administrations and routes will always be used to achieve the scientific objectives. Typically, the animals will not receive more than 3-4 intraperitoneal injections a week or 2-3 intravenous injections a week. On some occasions they may receive 2 injections on a given day through a combination of different routes.

For some more invasive procedures, general anaesthesia will be used to ensure the animals feel no pain.

For some experiments, mice will receive tumour cells through various routes; e.g. injected subcutaneously (under the skin) using a suitable vehicle or those spontaneously arising or induced. The tumours grow at their respective sites and can be measured over time using digital calipers, palpation or imaging. Mice are euthanised at a size that is deemed to not impact their normal behaviour. Throughout, the health status of the animal is the primary consideration used to define humane endpoints. Experiments will be terminated, or individual mice euthanised at the earliest signs of tumour-associated symptoms such as piloerection (goose bumps making the fur stand up), restricted movement, abnormal posture, abnormal gait, hunching, and/or weight loss up to, but not reaching, 20% body weight.

In many of our experiments, designed to measure immune changes, peripheral blood is taken from a superficial vessel. Pain from bleeding is controlled by suitable anaesthesia/analgesic with rapid healing and no long-lasting harm.

When the immune system is stimulated, for example with immunomodulatory agents, this can result in symptoms similar to those experienced during an infection (lethargy, fever etc). This can result in mice becoming less mobile, exhibiting pilo-erection etc. Typically, these symptoms are transient (first few hours) but can recur as the immune response develops (e.g. after several days) potentially resulting in further effects such as weight loss. These effects are therefore carefully monitored with mice euthanised if their symptoms become more severe or pass defined humane endpoints.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Experience from our current project licence has helped to determine the expected proportions of mice experiencing various severities for this project to be:

Sub-threshold - 0.6%

Mild - 64.5%

Moderate - 34.9%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst all attempts are made to reduce the use of animals by using in vitro methodology (in vitro experiments on human and mouse immune cells, explant studies and organotypic models), it is inevitable in work of this nature that in vivo investigation must be undertaken. The animal models detailed are critical to facilitate in vivo proof of concept and efficacy in an amenable and manipulable system, not possible in humans. We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro and so systemic in vivo studies remain fundamental to the study of complex tumour microenvironments, host factors and therapeutics. Further, advances, such as the availability of genetically altered mice and the development of more specific and sensitive techniques and reagents, continually permit a refinement and reduction in the types of experiments and the numbers of animals that are required (e.g. as highlighted by the organisation 'Fund for the Replacement of Animals in Medical Experiments', http://www.frame.org.uk) and this is something to which we remain committed. The increasing availability of genetically altered mice has allowed genetic dissection and more meaningful modelling of human disease.

Adult mice will be used for this study since they have a mature immune system and are the most appropriate species of mammal that have the following characteristics:

The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

Individual mice within a given inbred strain are considered genetically 'identical', thereby reducing variability and allowing valid conclusions to be drawn from experimental data;

Numerous tumour models have been established in mice that are strain specific;

Numerous strains of genetically altered mice have been developed (often replacing the mouse receptor (part of the cell which binds to a substance) with the human receptor) several of which will be central to this programme of work and have been developed by us to answer our specific aims and objectives. These mice will allow us to understand the mechanistic requirements for effective therapy in 'human' relevant in vivo contexts. These experiments are simply not possible in humans and will enable us to validate and extend our findings in a 'human' setting and to help inform the generation of novel agents for use in patients.

Which non-animal alternatives did you consider for use in this project?
We have made large strides to make more use of human material during the course of our current project licence. We now make extensive use of lymphocyte cones (blood cells collected from donors provided from NHS Blood Service) as a source of human immune cell populations for a range of immune assays and have built a range of clinical collaborations to allow us access to human tumour and healthy control material to study human cancer directly. Although powerful, these models are limited in both longevity and importantly complexity compared to in vivo systems and so we therefore still need to use appropriate mouse models to allow us to study our objectives in a whole-body system over a protracted time period and with facets such as biodistribution, pharmacokinetics and toxicity to be understood.

Further to this, and to try and better link between simple ex vivo culture assays and complex whole- body systems we have also embarked on a programme to develop appropriate human multi-cellular organotypic models for studying malignancy and the

impact of body composition on inflammation and cancer. To date we have published a model of lymphoma and demonstrated that we could model aspects of the tumour microenvironment and run limited antibody effector studies. We have also been developing a model of Osteosarcoma (a form of bone cancer) and have published a review of available models including; human organotypic, chorioallantoic membrane (a model using chicken eggs) and in vivo mouse models to widen awareness and interest in this area in the field. Finally, we have begun a project to develop a three-dimensional breast cancer organotypic model of obesity associated adipose inflammation. Here, we will again use a fully human system to investigate breast cancer development, progression and treatment response.

It is difficult to quantify the reductions in mouse numbers that these changes have facilitated given that the experimental systems are complementary and not interchangeable as they address distinct scientific questions. However, without question, access to and greater use of human material to address basic scientific questions enables us to replace animals in some circumstances and this should continue to grow in the future.

Why were they not suitable?

We need to investigate the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro. Each of the systems we have developed above, model different aspects of the immune response but none fully recapitulate the complexity and inter-relationships of a whole organism and so systemic in vivo models remain fundamental to our studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?
I have a current project licence and have been monitoring annual usage. This existing PPL has been interrupted by moving to a new animal facility and the coronavirus pandemic and therefore the numbers used do not represent those originally envisaged or planned. The number above represents predicted numbers based on current activity and taking into account reductions due to our shift to using primary human tissues, where possible, as well as likely refinements over the coming years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is always considered in light of how animal numbers can be reduced whilst ensuring meaningful and reproducible results. We are committed to reducing animal waste, implementing the three Rs, and maximising the reproducibility of research and so follow the PREPARE guidelines (https://norecopa.no/prepare) when considering our experiments.

As a first means to reduce the numbers of mice in our project, inbred mouse strains will be used to minimise variability of response and so allow reduced numbers to be used whilst delivering reproducible data. Related to this, we routinely use age- and sex-matched mice that have been bred in the same facility (ideally litter matched where control and GA mice are being compared) to minimise variability.

We have been using a range of tumour models for some time and so are well aware of the reproducibility of controls and appropriate mouse numbers required in tumour growth and therapy experiments. Where new models or treatments are introduced, we will first confirm expected humane endpoints and then perform pilot studies to inform the design of larger studies, including dosing regimens, as well as to monitor for any signs of adverse events. For example, 2 mice per group would be inoculated with a new tumour at different cell numbers to establish tumour growth kinetics etc., even where data exists in the literature, as we are cognisant of the impacts of the local environment, microbiome etc. Similarly, a single mouse would first be treated with a new experimental treatment to judge safety, immune response etc, before proceeding to larger studies.

We are also cognisant of ensuring we minimise experimental bias. Accordingly, in experiments where we implement more subjective humane endpoints as part of a tumour therapy experiment (e.g. abdominal palpation as opposed to caliper measurements), an experienced and treatment-group blinded animal technician is consulted regarding outcome.

Where we have useful data (from historical or pilot experiments), power analyses will be used to help guide the optimal numbers of mice needed for each experiment, taking into account expected magnitudes of impact. Power analysis is performed using the PS: Power and Sample Size Calculation programme: www.biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize):

For immunotherapy experiments, where we use inbred strains of mice, intra-group variability is reduced. From experience, we have found that we can use considerably fewer animals per group, usually 5, to detect such a difference at the 5% significance level in many of our models (e.g. EG7, MC38, B16-OVA and TC-1).

For monitoring immunological responses we typically use 5 mice per group for each experiment. From our experience, this number of mice/group allows us to identify with a statistical power of 95% a 3-fold difference in the number of CD8 T cells between 2 groups at the 5% significance level.

Therefore, the proposed mechanistic studies will be performed with experiments utilising groups of 5 mice, in a standard vehicle versus drug manner. We aim to test the candidate antibodies and immune modulators (identified by prior human and murine in vitro experiments) for efficacy in a selection of tumours. Throughout, replicate experiments will be performed to ensure reproducibility and when appropriate studies may be combined to increase sample size. To assess animal survival, endpoints will be plotted against time for each group in an experiment and the differences between groups will be analysed by appropriate statistical tests.

Should substances or tumour models be used for the first time, pilot studies will be performed on individual mice and humane endpoints established as indicated above. Substances will be administered and monitored after 1, 4 and then 24h for adverse effects before proceeding; a dose escalation schedule conforming to accepted practice will be

used should adverse effects be predicted. When agents are used for the first time in the laboratory, their dose will be based on previous published data whenever possible.

When new tumour lines are introduced they have to go through quarantine to ensure safe introduction into the animal facility. As an additional measure of reduction, we use this period to also monitor growth and humane endpoints reducing the need for additional animal use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice used across experiments are inbred thereby minimising intra-group variability and allowing reduced mouse numbers for experiments (we also, wherever possible, when breeding GA strains will use GA-ve littermate controls when available). Experiments are always designed with the fewest animals consistent with obtaining statistically valid results. We have performed Power analysis and our own extensive experience with these models to determine the numbers of mice required to deliver statistically significant results. Where appropriate, small pilot experiments are carried out to determine factors such as dose or route of administration.

Where multiple inter-relating parameters are to be evaluated, to prevent use of excess mice, we will employ higher dimensional analysis tools. Significant technological advances have enabled more information to be obtained from one individual mouse than was previously possible (e.g. using multi- parameter flow cytometry and RNA-seq technology), enabling multiple parameters to be assessed simultaneously from small samples. These technologies thereby facilitate longitudinal studies and reduce the need to cull multiple mice at different time points to sample from the spleen for instance. For example, we have largely moved from 3 to 8 and 16 colour flow cytometry, reducing the sample input requirements accordingly.

During the course of our current PPL, we have instigated a policy that new in vivo experiments must be detailed on a ‘study-plan’ detailing: the aims, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, and appropriate risk assessments. This has to be submitted to myself and the NACWO before an experiment is allowed to begin. An example of this document (Figure 1) is shown below:

Home Office

WT COVID 19 Contingency BRF Experimental Study Plan – Wild Types

PPL: **Tel No:**	**PI:** **Tel No:**	**PIL Holder:** **Contact number:** **Secondary PIL:** **Telephone number:**	**Are all required competencies required to carry out this experiment up to date?** Yes/No **Have you carried out the below procedures you intend to do for this experiment within the last 12 months?** Yes/No **List:** Competencies checked by (BRF Staff):		
Protocol number/title	**Grant/subproject code work will be charged to:** **Severity limit:** **Expected Severity of Overall Experiment:** **Sch1 (Only)** Yes/No **ERGO No**		**Strain/Genotype**	**Age (state usable age range):**	**Number of animals**
Experiment Identifier	**Start date:**			**Expected end date:**	
Objective of experiment & which objective of the PPL this links to (and how)					
Tumour Immunotherapy Yes/No	If *Yes*, which tumour and route	Humane end-point (as described in PPL, not Sch 1)			
Any cells other than tumour Yes/No	If *Yes*, cell type and route				

Administration of test reagents / surgery / anaesthesia / irradiation (or other procedural steps)	PIL/Delegated PIL?	Reagent(s) and vehicle	Route	Dose/Expected frequency	Post procedure monitoring (Dates, times and person)

Is this a new reagent? Yes/No	If *Yes*, describe
Are any known adverse effects/risks associated with this reagent? Yes/No	If *Yes*, describe. Have you made any delegated PIL aware of these? Yes/No

Is there a completed risk assessment in PCU? Yes/No	Confirm completed risk assessment reference number. (Please name and give unique numbers for any RAs, please also generate/provide any RAs if there isn't a generic one already created) :			
Are you administering materials that could contain rodent pathogens (cell lines, antibodies etc.)	If *Yes*, have they been screened? If not, where are they obtained from? •			
Monitoring/sampling method To include dates and times of visits. To be arranged with BRF staff	Tumour measurement	Palpation	Blood sampling:	Other:

Figure 1. Study Plan Template

This has facilitated a more detailed dialogue between PPLh and PILh prior to work and if necessary a discussion about the number of mice being used. More recently this process has included approval by representatives of the AWERB committee. This serves to ensure animal numbers are optimised.

Other optimisations to reduce animal use include:

Tumour cells being stored frozen when possible to prevent mice being used to passage tumour in vivo.

Consideration of freezing additional animal tissues (whole and as single cell suspensions) when an animal is sacrificed to provide controls for staining in different immune situations, relieving the need to cull specific mice for this comparative purpose.

Similarly, harvesting tissues; blood (for serum production), spleens (for lymphocytes) and bone marrow (to generate macrophages) when appropriate mice are culled for other purposes.

The in vitro use of immune cells isolated from mouse tissues offers several benefits and aids in optimising number of animals used for in vivo experiments. Importantly, this strategy is in line with the 3Rs. Due to the relatively low numbers of cells required to perform in vitro assays, such experimental approaches allow the screening of a high number of drugs or combination thereof, with potential for in vivo activity, while minimising the number of animals required. Furthermore, they allow refining doses, and identifying potential mechanisms of action and adverse reactions, prior to in vivo testing. In addition, in vitro assays using mouse immune cells represent a powerful reductionist approach to study their activation. They allow the isolation and analysis of distinct cell subsets, and the direct assessment of drugs on each individual cell population, otherwise difficult to accomplish.

For monitoring immunological responses in vitro, we generally use 1 mouse per experimental setting with 3 technical replicates and repeat the experiment two to three times. From our experience, this number of mice allows us to identify with a statistical power of 95% a 1.5 fold difference in immunological responses (eg. cell proliferation, upregulation of activation markers, cytokine expression, phagocytic uptake) between the control and treatment arms at the 5% significance level.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice for these studies as they are the most appropriate species of mammal that have the following characteristics:

The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

Individual mice within a given inbred strain are considered genetically ‘identical’, thereby reducing variability and allowing valid conclusions to be drawn from experimental data with as few animals as possible.

Numerous tumour models have been established in mice that are strain specific;

Several strains of genetically altered mice have been developed such as those expressing tumour antigens of interest (hCD20, HER2 etc.) and mice with genes of interest removed or added in order to elucidate immune mechanisms e.g. mice in which the mouse Fc gamma receptors have been replaced with their human counterparts. If required, we will generate new human knock-in (KI) mice to enable us to validate our findings in a ‘human’ setting and to help generate novel agents for use in patients as we have done with our unique human stimulator of interferon gene (STING) knock-in mouse model.

When evaluating immune-modulatory and immunotherapy approaches, we begin with in vitro evaluation and progress to pre-clinical animal models when sufficient promise is obtained. Our in vitro studies may include assays to determine the ability of reagents to kill or affect the growth or survival of target cells, and to recruit immune effector mechanisms. Where appropriate, we will first establish effects on in vitro cell lines and ex vivo primary material. However, in vitro lines and primary cells become adapted to cell culture conditions and are not present in a complex multicellular tissue/organ and therefore do not best recapitulate in vivo responses, hence the need for animal experiments.

In order to understand the mechanistic requirements for effective immunotherapy, cells may be obtained from WT or genetically altered mice with deficient/altered immune effector systems e.g. Fc gamma receptors or complement knock-out mice deficient in key effector molecules. For those reagents that show promise in vitro, we will determine if this translates into an in vivo effect using the most appropriate models. Typically, we would first examine reagents in the absence of tumour or other 'altered' host factors (e.g. increased fat mass), to assess immune-modulation, without unnecessarily increasing the welfare impact to animals by adding to the cumulative harm from further interventions and/or activities of the tumour. We ensure humane end-points are established that minimise the harm to the animal without compromising the accuracy of the experimental data.

Why can't you use animals that are less sentient?

Our work largely involves study of the immune system, which is a complex, multi-faceted, interacting system that is spread throughout the body and organ systems. The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapy strategies and their mechanisms in these animals - less sentient species have very different immune systems. More immature animals also do not display mature immune systems (the immune system becomes educated with age and development) that we seek to investigate and understand for clinical benefit. Anaesthesia reagents frequently interfere with multiple bodily functions which would alter the immune response and cannot be used for the extended periods required (days to weeks) to follow immune responses induced and so are not routinely used unless required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continually review our procedures and if unnecessary harms are identified or can be reduced, modified protocols are implemented through discussion with the NACWOs, PPL holder, academic lead for the BRF and named vet as required. We also seek to ensure the best housing and conditions for the animals are provided.

Over the course of my current PPL we have instigated the changes below that demonstrate our commitment to refinement:

We have moved to a new purpose-built animal facility that is more conducive to high quality in vivo research utilising a 'barrier' system meaning that all materials coming into the unit are clean and enter via a dedicated positive-pressure pass-through hatch. All personnel entering must now change into scrubs, and pass through an air-shower on entering (and leaving). The new facility also houses a separate quarantine facility that is no longer inside the main unit, but attached and accessible separately, with dedicated technicians only permitted.

All new strains of mice entering quarantine undergo rederivation before offspring are allowed in the main unit. All new cell lines must be screened in mice in quarantine, and serum from hosts/sentinels assessed for the presence of pathogens before cells are allowed to enter the main unit. All mice in our new facility are housed in individually ventilated cages. These changes minimise the risk of infection with obvious potential implications for the welfare of the animals and the quality of the research. In addition, the IVCs permit less disturbance to the animals as an additional refinement.

We have also implemented a number of new policies to improve welfare and reduce adverse experiences. All mice undergoing an injection now undergo a 'second check' by the person performing that procedure, within 30 mins - 4 hours. This was instigated to prevent animal welfare impact should an unanticipated adverse event occur. If a procedure is delegated to a technician, this second check also incorporates a 'positive handover' between the technician and the PILh to transfer the responsibility for the mice back to the PILh to ensure it is always clear where responsibility for welfare ultimately lies.

In response to changing guidelines a number of further and specific refinements have been put in place, including:

CO2 entering a chamber during schedule 1 culling, now enters from the top rather than from the bottom in line with current best practice. Flow rate is 20% of chamber volume/min. 3 min dwell time, where the CO2 is stopped but the chamber remains untouched so they remain in maximum CO2. Nb: the machine is serviced annually to check that it is still within parameters and we check that there is adequate CO2 pressure before starting and stay in the room during the CO2 delivery.

Mice are handled by ‘cupping’ or by gently moving them using a dedicated tunnel, prior to any restraint to ensure mice are calm and habituated to the person performing injections, serving to reduce anxiety when handling mice.

Instigated a policy that needles are only to be used once. This is to prevent mice receiving an injection with a blunted needle. Initially during the early part of this PPL the refinement was for needles to be used less than 5 times, but latterly we implemented the new single use policy unless specific justification is provided that is approved by BRF management committee.

We have transitioned towards venesection as our primary means to take blood samples instead of tail tipping. While this is not always possible (for instance when mice are receiving concurrent intravenous injections) new PILh are now trained in venesection and established PILh who regularly bleed mice have undergone re-training. Although retraining was delayed during COVID-19 restrictions and due to staff shortages and altered work-patterns/shifts, this has recently been re-initiated.

During the course of this PPL, we instigated a policy that new in vivo experiments must be detailed on a ‘study-plan’ (see Figure 1) and encompass the aims and how they address the PPL objectives, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, welfare measures and monitoring employed and appropriate risk assessments. This study-plan has to be submitted to the PPL and then responsible NACWO for approvals prior to experiment commencement. This has ensured that there is evidence of an appropriately detailed dialogue between PPLh and PILh prior to work and as necessary enabled a discussion about the number of mice used and procedures administered.

We also have a new system that records training competencies for individual PILh to ensure that training on all procedures is renewed every 3 years. Training is conducted by dedicated trainers using Detailed Operating Procedure (DOP) forms written specifically for this purpose. As PPLh I have access to these records, and reference is made to them on the ‘study plan’ (see Figure 1) ensuring that PILh also review their records regularly.

We have regular BRF Users meetings (approx 3 times per year) to discuss issues that arise within the animal facility and these are well attended by the personal licence and project licence holders, named animal care and welfare officers, animal technicians, home office liaison and compliance officer, named veterinary surgeon and establishment licence holder. Any issues and incidents that PILh need to be aware of are discussed at this meeting. We have also embarked upon a strategy of ensuring that PPLh and PILh undergo regular refresher training at least every 3 years to ensure that they remain abreast of changes to best practices and aware of their responsibilities under ASPA.

When possible, genotyping protocols for new Tg mice have been refined to allow PCR only protocols for screening, reducing the need to bleed mice prior to experimentation. Moreover, some existing transgenic strains (e.g. human CD20 transgenic) that were

previously screened via blood are now done so by PCR from ear notches reducing the number of procedures the mice undergo.

We have also adopted the practice of transferring male mouse nesting material, not substrate, to minimise male mouse aggression (the nest has calming pheromones in it, but the sawdust aggravates aggression as it holds the testosterone).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are a number of detailed publications and guidelines for the welfare and use of animals in cancer research that provide excellent guidance on the methodologies, study design and best practice that we will follow and adapt as appropriate to our research (Guidelines for the welfare and use of animals in cancer research: Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642).

We will continue to follow the ARRIVE guidelines https://arriveguidelines.org/ which provide a checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition, we will follow and consult NORECOPA https://norecopa.no/3r-guide : Norway's National Consensus Platform for the advancement of the 3Rs (Replacement, Reduction and Refinement associated with animal experiments) database platform and PREPARE https://norecopa.no/prepare (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best models for our research.

Finally, regular communication within our research group and communication with our peers at seminars and conferences ensures we're aware of any new or updated best practice. Best practice information is also disseminated by our Named Information Officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have regular BRF Users meetings (approx 3 times per year) to discuss issues that arise within the animal facility and these are well attended by the personal licence (PILh) and project licence (PPLh) holders, named animal care and welfare officers, animal technicians, home office liaison and compliance officer, named veterinary surgeon and establishment licence holder. Any issues and incidents that PILh need to be aware of are discussed at this meeting. Further to this, this platform is used to disseminate information from ASRU, NC3Rs and other organisations and to provide details of training opportunities to enhance welfare and research practice. We have also embarked upon a strategy of ensuring that PPLh and PILh undergo regular refresher training at least every 3 years to ensure that they remain abreast of changes to best practices and aware of their responsibilities under ASPA. These advances are further communicated through our active team of NACWOs and regular email updates. We will also stay up to date with specific cancer groups, databases and alternatives (NC3Rs) for cancer models such as: https://resources.researchanimaltraining.com/faqs/breast-cancer- research-alternatives-database; https://data.jrc.ec.europa.eu/dataset/352f7dfd-05cf-434b-a96a- 7e270dc76573.

168. Breeding and maintenance 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

Key words

Mouse, Breeding, Transgenic

Animal types	Life stages
Mice	juvenile, adult, pregnant, aged, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this work is to, breed and maintain mice with genetic alterations and supply them for research into the control of disease, ill-health or abnormality and/or the study of normal and abnormal physiology, biology or behaviour. They will be used for the discovery and development of new cancer treatments and prevention of this 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?

One in two of us will personally experience a positive diagnosis of cancer in our lifetime and cancer research is crucial to improve the prevention, treatment and detection of these cancers. Genetically altered mice are valuable animal models that contribute to the elucidation of a wide range of biological processes and diseases such as cancer. Although

in vitro approaches provide critical data, the use of animal models is essential to understand the very complex scenario of this disease.

What outputs do you think you will see at the end of this project?

A supply of genetically altered and non-genetically altered mice for use in research into cancer mechanisms and treatment.

Who or what will benefit from these outputs, and how?

A single centralised breeding project licence, managed by an individual with specialised technical expertise in the required breeding methodologies and with stringent colony management is administratively efficient and has anticipated welfare benefits.

These are:

Reduction in animal numbers by effective liaison with end-user projects, to ensure the appropriate strains of the desired specification are bred (best model for the disease areas), with minimal wastage and sharing of animal lines and/or tissues by several research programmes.

Reduced transport time for the animals, as fewer animals have to be brought in.

High health status, with direct benefit on welfare and science. The standard of facilities available for the work is high, and reflects a good level of investment. Secondary benefits will also be delivered through assisting other project licensees in meeting their objectives.

Under the previous licences there been a proven record of efficient generation and breeding of genetically altered animal strains and this project will continue and improve the provision of high quality animals to the researchers.

The output of the programme will be used by the scientists at the Establishment in both in vitro and in vivo studies aimed at understanding the processes involved in cancer initiation and metastases, as well as in the evaluation of newly discovered cancer targets in vivo before taken further in clinical trials.

How will you look to maximise the outputs of this work?

Myself and technicians in my team will hold regular meetings with and communicate as required with the institute group leaders (principal investigators) and scientists to ensure transgenic breeding is managed effectively and excess stocks are kept to a minimum. We will use proprietary software to manage the colonies efficiently. Where mouse strains are not required for a long time embryos / sperm of those mice will be preserved by freezing.

Species and numbers of animals expected to be used

- Mice: 90,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Researchers have studied laboratory mice as models of human cancer for many years and there are many standard protocols, methods and reagents that have been optimised for use with mice. Their use as cancer models has already provided exceptional insight into the biology and genetics of human cancers. Although it is possible to identify genes and targets that may cause susceptibility to a certain disease in vitro this can often only be fully investigated in an animal model that has been manipulated to study the gene effect. Without animal models it is impossible to determine what effect these changes will have on a whole living system. In vitro assays cannot adequately model the complete array of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes.

The majority of the research projects that we support require the mice to be adult at the time of tissue collection or enrolment into experimental protocols, in order to reflect typical ages of cancer development in humans. Some particular projects may necessitate the use of fetal, embryonic or immature mice, either because the processes being studied are developmental ones that have a relevance in cancer, or because the combination of transgenes is lethal at later stages of development.

Typically, what will be done to an animal used in your project?

Genetically altered animals will be bred under this licence in order to understand further different processes associate with cancer. The various steps involved will be:

Mice will be bred for research purposes. Some of the mice will be genetically altered to help address questions in cancer research and so may have phenotypic characteristics that require more regular monitoring. Animals will be monitored for unpredicted adverse effects. All procedures will be undertaken by trained, competent people.

Mice that are no longer going to be used will be humanely killed following the accepted protocol. No mice with genetic disabilities exceeding moderate severity will be bred on this licence.

What are the expected impacts and/or adverse effects for the animals during your project?

Some of the mice bred with alterations of genes for the purposes of investigating cancer mechanisms may display clinical symptoms that require regular and frequent monitoring. These potential clinical symptoms include: sub-cutaneous / abdominal masses; rectal prolapse; swollen eyes; skin spots; hairlessness; hind-limb issues. During the course of the previous five years these symptoms have only been seen rarely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The proportion of mice that are anticipated to display clinical signs resulting from gene alterations that lead to a moderate severity rating is, based on previous experience less than 1%. The proportion of animals that are expected to display clinical signs resulting

from gene alterations that lead to a severe severity rating is, based on previous experience less than 1%.

All mice will be managed so that they do not suffer if developing early signs of the clinical symptoms.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The different animal models maintained and bred under this license will integrate the complete range of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes, focusing on cancer. This cannot be achieved without the use of animals.

The mouse is one of the model organisms that most closely resemble humans. The human and mouse genomes are approximately the same size, and display an equivalent number of genes, which are functionally conserved. Definitively, mouse models are important for placing the findings of in vitro studies or correlative analysis of human samples into an appropriate and meaningful in vivo context

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives have been excluded by the Project Licences for which we are providing mice. The reason why is that cancer is a multi-faceted disease dependent upon the interaction of many different cell types interacting in a dynamic three-dimensional environment.

Why were they not suitable?

The reasons for not being suitable was provided by the individual Project Licences for which we are providing mice.

In general the study of cells in culture (in vitro) and less sensitive organisms 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 to allow us to investigate the impact of the relationship between the immune systems in the promotion of cancer cell growth and spread.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design

studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimations of numbers of animals required are based on actual numbers used in previous project licences for breeding and predicted future requirements of Project Licences being supplied with mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design will be covered under separate experimental PPLs, the mice will be transferred to these as required. Breeding programmes will be agreed in advance and regularly reviewed to optimally meet anticipated demand. Breeding programmes will be optimised wherever possible to produce only the required genotype.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Freezing of eggs / embryos and sperm will be carried as routine by a separate team responsible for cryopreservation of mouse strains, working under a different project licence.

Archiving of mouse strains will avoid unnecessary need to maintain colonies by continuous breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Researchers have used laboratory mice as models of human cancer for many years. There are several reasons that make this rodent the best model to use: the mouse has a short reproductive cycle, large litter sizes, is easy to maintain and can be readily shipped from breeding facilities to research locations. Only natural mating methods will be used and these are not anticipated to cause pain, suffering, distress or lasting harm.

Why can’t you use animals that are less sentient?

The mouse is the least sentient mammal for which there are many well established laboratory protocols and a significant amount of background data. A mammalian model is required in order to more closely model the processes relevant to cancer that are seen in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All mice in the facility are regularly monitored in order to detect any potential welfare issues.

In addition to usual daily checks all breeding females are checked for pregnancy and this is flagged when detected, so that conditions such as dystocia can be monitored for. Litters are noted as soon as they are detected, but to minimise stress new litters are not usually disturbed to count pups for the first couple of days after birth.

Breeding performance (including factors such as litter sizes, lost litters, aggressive behaviour and age) and stock levels are monitored by the technicians who are responsible for each line, who then communicate with users as needed regarding potential replacements or other changes required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Breeding and Colony Management Best Practices. https://nc3rs.org.uk/breeding-and-colony- management. Wells DJ, Playle LC, Enser WEJ, Flecknell PA, Gardiner MA, Holland J, Howard BR, Hubrecht R, Humphreys KR, Jackson IJ, Lane N, Maconochie M, Mason G, Morton DB, Raymond R, Robinson V, Smith JA and Watt N. Assessing the welfare of genetically altered mice. Laboratory Animals (2006) 40, 111–114.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Project Licence holder and other team members will attend meetings such as the annual meetings of Laboratory Animal Science Association (LASA) and Institute of Animal Technology (IAT), as well as smaller national meetings such as the National Centre for 3Rs (NC3R) Understanding Animals in Research (UAR) meetings. The Project Licence holder will stay abreast of relevant literature. Relevant advances will usually be initially implemented in the form of pilot projects and, where technically feasible, will then be implemented more widely.

169. Novel immunotherapeutic strategies to treat Cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, antibody, immunotherapy, immune-modulation, immune system

Animal types	Life stages
Rats	adult
Mice	adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to explore the utility of new anti-cancer reagents for use in the clinic. The specific objectives are:

To produce and characterize new anti-cancer reagents.

To determine how these reagents work and how they can be improved.

To develop strategies to promote/modulate the immune response to cancer and to understand how this happens

To understand how tumours form with the aim of developing reagents that can stop them.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In 2020 there were more than 19 million new cases of cancer diagnosed alongside 10 million cancer- related deaths worldwide. With such a high worldwide healthcare burden, the need for effective anti- cancer treatments is paramount. Although the conventional treatments of surgery, chemotherapy and radiotherapy have been effective in the treatment of certain cancers and in improving outcomes for many patients, frequently these treatments fail and tumours come back. Understanding why tumours form and how they overcome treatment is critical for developing more effective treatments. In the last 2 decades it has become increasingly clear that tumour cells interact with the host immune system. This knowledge has led to a huge surge in the field of immunotherapy – which studies how the immune system can be leveraged to treat diseases including cancer. The idea is to redirect or reinvigorate the immune response against cancer, in therapies that are highly specific and effective with the potential to prevent tumour recurrence, in the same way we become immunised against measles or mumps.

What outputs do you think you will see at the end of this project?

Immunotherapy offers the potential for treating a wide range of cancers in a highly focussed and specific way. The immune system has the amazing ability to distinguish pathogens from the host and can do the same in recognising, detecting and removing cancer cells.

The most successful immunotherapy drugs explored to date are monoclonal antibodies (abbreviated as mAb). Using these cancer-specific reagents, we can direct the patient’s own immune system to destroy unwanted cells while leaving normal tissue mostly untouched. Consequently, unlike conventional treatment, mAb treatment will not be associated with long-lasting toxicity. In addition, it has the potential through what is termed "immunological memory", which forms the basis of vaccines, to mean that the patient will continue to recognise and kill their tumour cells on an ongoing basis, reducing the potential for relapse. In this project we will deliver these benefits through the development and characterization of new immunotherapeutic reagents and treatment strategies.

Potential benefits from our work also arise from our ability to develop new combinations of treatments in which mAb are given alongside conventional and other emerging methods, including other mAb. The ultimate aim is to develop and /or improve treatment options for patients with a range of cancers.
However, this can only be achieved through a far better understanding of the interaction between the cancer cells and the immune system.

Therefore, during our programme of work we anticipate discovering and patenting new reagents and approaches for cancer treatment alongside our partners such as Cancer Research UK and Biotech/pharmaceutical companies. Several reagents developed under our current and previous licences have been or are already in clinical trials.

Who or what will benefit from these outputs, and how?
There will be multiple beneficiaries from our studies. In addition to those cancer patients who may benefit from our discoveries in the longer-term, we will publish our results in peer-

reviewed journals and present at scientific conferences in the short-term. This manuscripts will be available on eprints 3 months after publication. Our data will be of interest to scientists, pharmaceutical companies and clinicians - helping them to make further progress towards deeper understanding and ultimately hopefully new and better cancer drugs in the future. Moreover, often our aim in cancer is to stimulate the immune system to attack the cancer cells and the same is often true in infection. Therefore, the principles established during these studies should also be applicable to immunotherapy against infectious diseases for both clinical and veterinary applications. Furthermore, the opposite effect is required in the treatment of autoimmune disorders (a condition in which the body's immune system mistakenly attacks healthy tissues) and so drugs that we develop, should they prove to be inhibitors rather than stimulators (reduces the effect rather than boosts it), or understanding that we gain that is not of use in the treatment of cancer, may well be of use for the treatment of these diseases and for the benefit of human health.

How will you look to maximise the outputs of this work?

We will maximise our outputs by publishing our findings widely and freely. Where possible we publish in open access journals, with full disclosure of the associated raw data, enabling our studies and results to be accessible to all. We have an excellent track record in publishing our findings (>180 to date with myself as an author), including those where approaches were not ultimately successful. In addition, almost all of our work is performed in collaboration with other academic groups and/or Biotech/Industry partners and so shared knowledge and expertise further maximises our outputs.

Species and numbers of animals expected to be used

- Mice: 14304
- Rats: 16

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Whilst all attempts are made to reduce the use of animals by using in vitro methods (e.g. isolating and performing experiments on various types of blood cells that are an essential part of the immune system outside of the body) and explant studies (using tissue grown in a culture medium out of the body), it is inevitable in work of this nature, assessing the immune system, that in vivo investigation must be undertaken. Animal models are critical to facilitate in vivo (in a living animal) proof of concept and efficacy in an amenable and manipulable system, not possible in humans. We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro (in an artificial environment) and so systemic in vivo studies remain fundamental to the study of new complex tumour microenvironments and therapeutics.
Rodents (principally mice) will be used for this study since they are the most appropriate species of mammal that have the following characteristics:

The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

Individual mice within a given inbred strain are considered genetically 'identical', thereby reducing variability and allowing valid conclusions to be drawn from experimental data.

Numerous tumour models have been established in mice that are strain specific; although none fully capture every aspect of the human disease, models can be selected that allow specific questions to be addressed to facilitate translation.

Numerous strains of genetically altered mice have been developed. These genetically altered mice allow more meaningful modelling of human disease. These experiments are simply not possible in humans and will enable us to validate and extend our findings in a 'human' setting and to help inform the generation of novel agents for use in patients.

Mice will be used as they possess a mammalian immune system, reflecting that of humans.

For the raising of certain monoclonal antibodies we will use rats, as they allow the generation of antibodies to mouse targets (not possible to raise in mice) to serve as tool reagents and for dissection of mechanism of action prior to development of human antibodies.

Typically, what will be done to an animal used in your project?

A series of different types of experiments are proposed which enable us to introduce and measure immune-modulatory agents (including antibodies) and assess their effects on the immune system as well as tumour cells.

In experiments designed to develop new antibody drugs, mice will be injected with material to enable them to mount an immune response over a period of weeks, before the mice are euthanised and their B cells isolated to make hybridomas.

In experiments designed to measure effects on the immune system, mice will be injected with material (such as an antibody) and then the immune response measured by taking peripheral blood (much as we do for patients) and measuring changes in the immune cells (typically over a period of days to weeks). A proportion of mice will be euthanised and their tissues examined to understand how the immune response is developing in the different organs. These experiments are often done in the presence of a growing tumour, introduced earlier through injection, allowing us to assess infiltration of immune cells into the tumour after different treatments. A proportion of mice are then followed to measure tumour growth over time in experiments that may last 1-2 months, allowing us to determine if the treatments have cured the mice or delayed the tumour growth.

What are the expected impacts and/or adverse effects for the animals during your project?

We typically identify mice by ear notching - this is expected to result in only mild and transient pain with no healing problems.

Mice will be injected with various immunomodulatory substances using a combination of volumes, routes and frequencies. Injections will cause momentary needle stick pain minimised by using the needle of the smallest suitable gauge. The minimum number of

administrations and routes will always be used to achieve the scientific objectives. Typically, the animals will not receive more than 3-4 intraperitoneal (i.p.) injections a week or 2-3 intravenous (i.v.) injections a week. On some occasions they may receive 2 injections on a given day through a combination of different routes.

For some more invasive procedures, general anaesthesia will be used, to ensure the animals feel no pain.

For some experiments, mice will receive tumour cells through various routes; e.g. injected subcutaneously (under the skin) using a suitable vehicle. The tumours grow at this site and can be measured over time using digital calipers. Mice are euthanised at a size that is deemed to impact their normal behaviour, which may vary for different tumour models, based upon previous research.
Throughout, the health status of the animal is the primary consideration used to define humane endpoints. Experiments will be terminated, or individual mice euthanised at the earliest signs of tumour- associated symptoms such as piloerection (goose bumps making the fur stand up), restricted movement, abnormal posture, abnormal gait (movement when walking), hunching, and/or weight loss up to, but not reaching, 20% body weight.

In many of our experiments, designed to measure immune changes, peripheral blood is taken from a superficial vessel. Pain from bleeding is controlled by suitable anaesthesia/analgesic with rapid healing and no long-lasting harm. When the immune system is stimulated, for example with immunomodulatory antibodies, this can result in symptoms similar to those experienced during an infection (lethargy, fever etc.). This can result in mice becoming less mobile, exhibiting pilo-erection etc. Typically these symptoms are transient (first few hours) but can recur as the immune response develops (e.g. after several days) potentially resulting in further effects such as weight loss. These effects are therefore carefully monitored with mice euthanised if their symptoms become more severe or pass defined humane endpoints.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

During our current project licence the proportion of mice experiencing sub-threshold, mild, moderate or severe severities was as follows and we expect similar proportions in our new project licence:

sub-threshold 0.2%

mild 80.2%

moderate 19.6%
severe <0.1%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst all attempts are made to reduce the use of animals by using in vitro methods (in vitro experiments on various immune cells and explant studies using tissues grown outside of the body - see below), it is inevitable in work of this nature, involving the immune system that investigation in vivo (in an entire living organism) must be undertaken. The animal models proposed are critical to facilitate in vivo proof of concept and efficacy in an amenable and manipulable system, not possible in humans.

We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro and so systemic in vivo studies remain fundamental to the study of new complex tumour microenvironments and therapeutics. Further, advances, such as the availability of genetically altered animals and the development of more specific and sensitive techniques and reagents, continually permit a refinement and reduction in the types of experiments and the numbers of animals that are required (e.g. as highlighted by the organization 'Fund for the Replacement of Animals in Medical Experiments', http://www.frame.org.uk) and this is something to which we remain committed. The increasing availability of genetically altered mice has allowed genetic dissection and more meaningful modelling of human disease.

Specifically, mice will be used for this study since they are the most appropriate species of mammal that have the following characteristics:

The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

Individual mice within a given inbred strain are considered genetically 'identical', thereby reducing variability and allowing valid conclusions to be drawn from experimental data.

Numerous tumour models have been established in mice that are strain specific; although none fully capture every aspect of the human disease, models can be selected that allow specific questions to be addressed to facilitate translation.

Numerous strains of genetically altered mice have been developed, often replacing a mouse gene with its human counterpart, allowing us to study these interactions in vivo for the first time. For example, the human target of an antibody may replace the mouse counterpart, allowing antibodies directed against the human target to be tested. These experiments are simply not possible in humans and will enable us to validate and extend our findings in a 'human' setting and to help inform the generation of novel agents for use in patients.

Which non-animal alternatives did you consider for use in this project?

As a group, we have sought to increase our access to primary human material to reduce the requirement for animals wherever possible. We are now able to purchase lymphocyte "cones" from the local blood transfusion service and this enables us to perform

experiments using human peripheral blood lymphocytes. These cones are a natural by-product produced when people donate platelets, and contain many other immune cells of interest. We also have links with clinicians resulting in access to primary human tumours enabling us to investigate the effects of mAbs on human T cells infiltrating a tumour site in vitro. While we ultimately still need to use mice to study the influence of mAbs on a growing tumour and in a whole body system, better access to human material has enabled us to answer some clinically-relevant questions without the use of mice. It is difficult to accurately quantify the reductions in mouse numbers that this change has facilitated since the two experimental systems are not interchangeable but address distinct scientific questions. However, undoubtedly access to human material and the ethical clearance to address basic scientific questions enables us to replace animals in some circumstances.

In an attempt to help predict efficacy and toxicity of certain mAb we made use of the lymphocyte cones and have developed and adopted an in vitro assay using human cells as a replacement to mouse in vivo studies.

We are also evaluating in vitro protocols (phage display) to produce mAbs to our selected targets. This will reduce the number of animals used in the coming project licence, although these make up a very small proportion of the total animal usage.
Finally, we have also explored the use of organoid/ three-dimensional (3D) in vitro systems to study more tissue-relevant impacts of our immune interventions. Organoids and other 3D systems are typically small, self-organized 3D tissue cultures attempting to replicate the complexity of an organ/tissue, or to express selected aspects of it. Over a period of 6 years we were able to develop a 3D system to mimic certain aspects of a particular type of lymphoma microenvironment. Importantly, this model did not incorporate T cells, nor tumour-derived immune cells and so was unable to fully mimic the situation in lymphoma patients. It also represents a model of just a single type of lymphoma, with a similar amount of development required to produce equivalent models for other lymphoma sub-types and tumours. Therefore, as above we see these models as complementary to, rather than fully replacing the mouse models - with each addressing distinct scientific questions.

Why were they not suitable?

We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro. Each of the systems we have developed above, model different aspects of the immune response but none fully recapitulate the complexity and inter-relationships of a whole organism and so systemic in vivo models remain fundamental to our studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have a current project licence and have been monitoring annual usage. This new project licence (PPL) represents a reduction of almost 50% in comparison to our estimate from

our current PPL and takes into account our shift to use primary human tissues where possible as well as likely refinements over the coming years, alongside principal investigators within our research group holding their own project licences.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is always considered in light of how the animal numbers can be reduced whilst ensuring meaningful and reproducible results. We are committed to reducing animal waste, implementing the three Rs, and maximising the reproducibility of research and so follow the PREPARE guidelines (https://norecopa.no/prepare) when considering our experiments.

As a first means to reduce the numbers of mice in our project, inbred mouse strains will be used to reduce variability of response and so allow reduced numbers sufficient to deliver reproducible data. Related to this, we routinely use age- and sex-matched mice that have been bred in the same facility (ideally litter matched where control and genetically altered mice are being compared) to minimise variability.

We have been using a range of tumour models for some time and so are well aware of the reproducibility of controls and appropriate mouse numbers required in tumour growth and therapy experiments. Where new models or treatments are introduced, we will first perform pilot studies to inform the design of larger studies, including dosing regimen, expected humane endpoint etc, as well as to monitor for any signs of adverse events. For example, 2 mice per group would be inoculated with a new tumour at different cell numbers to establish tumour growth kinetics etc. even where data exists in the literature as we are cognisant of the impacts of the local environment, microbiome etc. Similarly, a single mouse would first be treated with a new experimental treatment to judge safety, immune response etc, before proceeding to larger studies.

We are also cognisant of ensuring we minimise experimental bias. Accordingly, in experiments where we implement more subjective humane end points as part of a tumour therapy experiment (e.g. abdominal palpation as opposed to caliper measurements), an experienced and treatment-group blinded animal technician is consulted regarding outcome.

Where we have useful data (from historical or pilot experiments), power analyses will be used to help guide the optimal numbers of mice needed for each experiment, taking into account expected magnitudes of impact. Power analysis is performed using the PS: Power and Sample Size Calculation programme: www.biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize):

For immunotherapy experiments, where we are using inbred strains of mice, intra-group variability is reduced. From experience, we have found that we can use considerably fewer animals per group, usually 5, to detect such a difference at the 5% significance level in many of our models (e.g. EG7, MC38, B16-OVA and TC-1).

For monitoring immunological responses we typically use 5 mice per group for each experiment. From our experience, this number of mice/group allows us to identify with a statistical power of 95% a 3-fold difference in the number of CD8 T cells between 2 groups at the 5% significance level.

Therefore, the proposed mechanistic studies will be performed with experiments utilising groups of 5 mice, in a standard vehicle versus drug manner. We aim to test the candidate immune stimulators (identified by prior human and murine in vitro experiments) for efficacy in a selection of tumours.
Throughout, replicate experiments will be performed to ensure reproducibility and when appropriate studies may be combined to increase sample size. To assess differences in animal survival between groups appropriate statistical tests will be used (e.g. Kaplan Meier curves will be analysed by Log rank test).

Should substances or tumour models be used for the first time, pilot studies will be performed on individual mice and humane endpoints established as indicated above. Substances will be administered and monitored after 1, 4 and then 24h for adverse effects before proceeding; a dose escalation schedule conforming to accepted practice will be used should adverse effects be predicted. When agents are used for the first time in the laboratory, their dose will be based on previous published data whenever possible.

When new tumour lines are introduced they have to go through quarantine to ensure safe introduction into the animal facility. As an additional measure of reduction, we use this period to also monitor growth and humane endpoints reducing the need for additional animal use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice used across experiments are inbred thereby minimising intra-group variability and allowing reduced mouse numbers for experiments. We also wherever possible when breeding Genetically altered strains will use their non Genetically altered (e,g, transgene negative) littermate controls when available. Experiments are always designed with the fewest animals consistent with obtaining statistically valid results. We have performed Power analysis to determine the numbers of mice required to deliver statistically significant results, although through experience we find we can often use smaller numbers of animals without sacrificing statistical significance as they are inbred strains. Where appropriate, small pilot experiments are carried out to determine factors such as dose or route of administration.

Where multiple inter-relating parameters are to be evaluated, to prevent use of excess mice, we will employ higher dimensional analysis tools. Significant technological advances have enabled more information to be obtained from one individual mouse than was previously possible (e.g. using multi- parameter flow cytometry and RNA-seq technology), enabling multiple parameters to be assessed simultaneously from small samples. These technologies thereby facilitate longitudinal studies (repeated observations of the same variables over time) and reduce the need to cull multiple mice at different time points to sample from the spleen for instance. For example, we are moving from 3 colour flow cytometry to 8 and more colour flow cytometry, reducing the sample input requirements accordingly.

Since the start of our current PPL, we have instigated a policy that new in vivo experiments must be detailed on a ‘study-plan’ detailing: the aims, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, and appropriate risk assessments. This has to be submitted to myself and the Named Animal Care & Welfare Officer (NACWO; person who is responsible for checking the

welfare of the animals) before an experiment is allowed to begin. An example of this document is shown below:

BRF Experimental Study Plan

<table>
<tr><td>PPL:

Tel No:</td><td>PI:

Tel No:</td><td colspan="2">PIL Holder:

Contact number:

Secondary PIL:

Telephone number:</td><td colspan="3">Are all required competencies required to carry out this experiment up to date?
Yes/No
Have you carried out the below procedures you intend to do for this experiment within the last 12 months?
Yes/No

List:

Competencies checked by (BRF Staff):</td></tr>
<tr><td colspan="2" rowspan="4">Protocol number/title</td><td colspan="2">Grant/subproject code work will be charged to:</td><td rowspan="4">Strain/Genotype</td><td rowspan="4">Age (state usable age range):</td><td rowspan="4">Number of animals</td></tr>
<tr><td colspan="2">Severity limit:

Expected Severity of Overall Experiment:</td></tr>
<tr><td colspan="2">Sch1 (Only)
Yes/No</td></tr>
<tr><td colspan="2">ERGO No</td></tr>
<tr><td colspan="2">Experiment Identifier</td><td colspan="3">Start date:</td><td colspan="2">Expected end date:</td></tr>
<tr><td colspan="2">Objective of experiment & which objective of the PPL this links to (and how)</td><td colspan="5"></td></tr>
<tr><td>Tumour Immunotherapy
Yes/No</td><td>If Yes, which tumour and route</td><td colspan="5">Humane end-point (as described in PPL, not Sch 1)</td></tr>
<tr><td colspan="2">Any cells other than tumour
Yes/No</td><td colspan="3">If Yes, cell type and route</td><td colspan="2"></td></tr>
<tr><td>Administration of test reagents / surgery / anaesthesia / irradiation (or other procedural steps)</td><td>PIL/Delegated PIL?</td><td>Reagent(s) and vehicle</td><td>Route</td><td>Dose/Expected frequency</td><td colspan="2">Post procedure monitoring (Dates, times and person)</td></tr>
<tr><td colspan="2">Is this a new reagent?
Yes/No

Are any known adverse effects/risks associated with this reagent?
Yes/No</td><td colspan="5">If Yes, describe

If Yes, describe.

Have you made any delegated PIL aware of these? Yes/No</td></tr>
</table>

Is there a completed risk assessment in PCU? Yes/No	Confirm completed risk assessment reference number. (Please name and give unique numbers for any RAs, please also generate/provide any RAs if there isn't a generic one already created) :			
Are you administering materials that could contain rodent pathogens (cell lines, antibodies etc.)	If *Yes*, have they been screened? If not, where are they obtained from? •			
Monitoring/sampling method To include dates and times of visits. To be arranged with BRF staff	Tumour measurement	Palpation	Blood sampling:	Other:

***Please confirm (Highlight) what BRF facility this work will be conducted in:**

***Please also indicate (Highlight) what further buildings/facilities will be used during this experiment:**

This form must be filled in and sent to the PPL holder and NACWO before the start of the experiment. Once reviewed by PPLh and NACWO this form will be considered by BRF Management Group.

This has facilitated a more detailed dialogue between PPL holder (PPLh) and personal licence holder (PILh) prior to work and if necessary a discussion about the number of mice being used. More recently this process has included approval by representatives of the Animal Welfare Ethical Review Body (AWERB) committee. This serves to ensure animal numbers are optimised.

Other optimisations to reduce animal use include:

Tumour cells being stored frozen when possible to prevent mice being used to passage tumour in vivo.

Consideration of freezing additional animal tissues (whole and as single cell suspensions) when an animal is sacrificed to provide controls for staining in different immune situations, relieving the need to cull specific mice for this comparative purpose.

Similarly, harvesting blood (for serum production), spleens for lymphocytes and bone marrow (to generate macrophages) when appropriate mice are culled for other purposes.

The in vitro use of immune cells isolated from mouse tissues offers several benefits and aids in optimising number of animals used for in vivo experiments. Importantly, this strategy is in line with the 3Rs. Due to the relatively low numbers of cells required to perform in vitro assays, such experimental approaches allow the screening of a high number of drugs or combination thereof, with potential for in vivo activity, while minimising the number of animals required. Furthermore, they allow refining doses, and identifying potential mechanisms of action and adverse reactions, prior to in vivo testing. In addition, in vitro assays using mouse immune cells represent a powerful reductionist approach to study their activation. They allow the isolation and analysis of distinct cell subsets, and the direct assessment of drugs on each individual cell population, otherwise difficult to accomplish.

Furthermore, having identified that the hIgG2 isotype is especially powerful for driving immune- stimulation in vivo, we are now following up on the molecular basis behind these findings with colleagues in Biology and Chemistry, using molecular simulations and other in silico approaches (reducing the need for animal experiments). We plan to develop prediction tools to guide our studies, further reducing the numbers of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rodents for these studies as they are the most appropriate species of mammal that have the following characteristics:

The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

Individual mice within a given inbred strain are considered genetically ‘identical’, thereby reducing variability and allowing valid conclusions to be drawn from experimental data with as few animals as possible.

Numerous tumour models have been established in mice that are strain specific;

Numerous strains of genetically altered mice have been developed such as those expressing tumour antigens or human receptors (part of the cell which binds to a substance) of interest (human CD20, human OX40 etc.) and mice with genes of interest removed or added in order to elucidate immune mechanisms e.g. mice in which a mouse Fc gamma Receptor (FcgR) has been replaced with its human counterpart. If required we will source new human knock-in (KI) mice to enable us to validate our findings in a ‘human’ setting and to help generate novel agents for use in patients as we have done.

When evaluating immunomodulatory and immunotherapeutic approaches, we begin with in vitro evaluation and progress to pre-clinical animal models when sufficient promise is obtained. Our in vitro studies may include assays to determine the ability of reagents to kill or affect the growth or survival of target cells, and to recruit immune effector mechanisms. Where appropriate, we will first establish effects on in vitro cell lines and ex vivo primary material. However, in vitro lines and primary cells become adapted to cell culture conditions and are not present in a complex multicellular tissue/organ and therefore do not best recapitulate in vivo responses, hence the need for animal experiments.

In order to understand the mechanisms operating, cells may be obtained from unmodified, wild type (WT) or genetically altered animals with deficient/altered immune effector systems e.g. FcgR or complement knock-out, mice deficient in key effector molecules. For

those reagents that show promise in vitro, we will determine if this translates into an in vivo effect using the most appropriate models. Typically, we would first examine reagents in the absence of tumour, to assess immunomodulation, without unnecessarily increasing the harm to animals by adding to the cumulative harm from further interventions and/or activities of the tumour. We ensure humane end-points are established that minimise the harm to the animal without compromising the veracity of the experimental data.

Why can't you use animals that are less sentient?

Our work largely involves study of the immune system, which is a multi-faceted, interacting system that is spread throughout the body and organs, linked by the vasculature. The cellular and molecular interactions of the rodent immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals - less sentient species have very different immune systems (e.g. zebrafish lack adaptive immunity). More immature animals also do not display mature immune systems (the immune system becomes educated with age and development) that we seek to investigate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continually review our procedures and if unnecessary harms are identified or can be reduced, modified protocols are implemented through discussion with the NACWOs, PPL holder, academic lead for the BRF and named vet surgeon (NVS) as required. We also seek to ensure the best housing and conditions for the animals are provided.

As examples, of our commitment to these aspirations over the last 4 years we have:

Moved to a new purpose-built animal facility. This unit is more conducive to high quality in vivo research utilising a 'barrier' system meaning that all materials coming into the unit are clean and enter via a dedicated positive-pressure pass-through hatch. All personnel entering must now change into scrubs, and pass through an air-shower on entering (and leaving). The new facility also houses a separate quarantine facility that is no longer inside the main unit, but attached and accessible separately, with dedicated technicians only permitted.

All new strains of mice entering quarantine undergo rederivation before offspring are allowed in the main unit. All new cell lines have to be screened in mice in the quarantine room, and serum from sentinels assessed for the presence of pathogens before cells are allowed to enter the main unit. All mice are now housed in individually ventilated cages (IVCs). These changes minimise the risk of infection with obvious potential implications for the welfare of the animals and the quality of the research. In addition, the IVCs permit less disturbance to the animals as an additional refinement.

We have also implemented a number of new policies to improve welfare and reduce adverse experiences. All mice undergoing an injection now undergo a 'second check' by the person performing that procedure, within 30 mins-4 hours. This was instigated to prevent animal welfare impact should an unanticipated adverse event occur. If a procedure is delegated to a technician, this second check also incorporates a 'positive handover' between the technician and the PILh to transfer the responsibility for the mice back to the PILh to ensure it is always clear where responsibility for welfare ultimately lies.

In response to changing guidelines a number of further and specific refinements have been put in place. These include:

that CO2 entering a chamber during schedule 1 culling, enters from the top rather than from the bottom in line with current best practice. Flow rate is 20% of chamber volume/min. 3 min dwell time, where the CO2 is stopped but the chamber remains untouched so they remain in maximum CO2. Nb: the machine is serviced annually to check that it is still within parameters and we check that there is adequate CO2 pressure before starting and stay in the room during the CO2 delivery.

that mice are handled by 'cupping' or by gently moving them using a dedicated tunnel, prior to any restraint to ensure mice are calm and habituated to the person performing injections, serving to reduce anxiety when handling mice.

a policy that needles are only to be used once. This is to prevent mice receiving an injection with a blunted needle. Initially during the early part of my current project licence (PPL), the refinement was to be used less than 5 times but last year we implemented a new single use policy unless specific justification is provided that is approved by the BRF management committee.

we have now transitioned towards venesection (tail pricking) to take blood samples instead of tail tipping. While this is not always possible (for instance when mice are receiving concurrent i.v. injections) new PILh are now trained in venesection and established PILh who regularly bleed mice have undergone re-training.

During the course of this PPL, we instigated a policy that new in vivo experiments must be detailed on a 'study-plan' that encompass the experimental aims and how they address the PPL objectives, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, welfare measures and monitoring employed and appropriate risk assessments (see study-plan template in Reductions above). This study-plan has to be submitted to the PPL and then responsible NACWO for approvals prior to experiment commencement. This has ensured that there is evidence of an appropriately detailed dialogue between PPLh and PILh prior to work and as necessary enabled a discussion about the number of mice used and procedures administered.

We also have a new system that records training competencies for individual PILh to ensure that training on all procedures is renewed every 3 years. Training is conducted by dedicated trainers using Directly Observed Practical (DOP) skills forms written specifically for this purpose. As PPLh I have access to these records, and reference is made to them on the 'study plan' (see above) ensuring that PILh also review their records regularly.

An additional refinement implemented has been to modify the humane endpoint for our CT26 tumour experiments; reducing the tumour size from 400mm2 to 300mm2 for most experiments; this allows us to obtain sufficient scientific information whilst reducing the risk of tumours impairing the welfare of the animal such as through impaired movement. When possible, genotyping protocols for new transgenic (Tg) mice have been refined to allow PCR only protocols for screening, reducing the need to bleed mice prior to experimentation. Moreover, some existing transgenic strains (e.g. human CD20 transgenic) that were previously screened via blood are now done so by polymerase chain reaction (PCR; a technique for amplifying genetic material useful in transgenes) from ear notches reducing the number of procedures the mice undergo.

We have also adopted the practice of transferring male mouse nesting material, not substrate, to minimise male mouse aggression (the nest has calming pheromones in it, but the sawdust aggravates aggression as it holds the testosterone).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are a number of detailed publications and guidelines for the welfare and use of animals in cancer research on cancer models that provide excellent guidance in the methodologies, study design and best practice that we will follow and adapt as appropriate to our research (Guidelines for the welfare and use of animals in cancer research Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642).

We will follow the ARRIVE guidelines https://arriveguidelines.org/ which provide a checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition, we will follow and consult NORECOPA https://norecopa.no/3r-guide: Norway's National Consensus Platform for the advancement of the 3Rs (Replacement, Reduction and Refinement associated with animal experiments) database platform and PREPARE https://norecopa.no/prepare (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best models for our research.

Finally, regular communication within our research group and communication with our peers at seminars and conferences ensures we're aware of any new or updated best practice. Best practice information is also disseminated by our Named Information Officer.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have a very active Biomedical research facility (BRF) users group, meeting approx 3 times per year to discuss issues that arise within the animal facility. These are attended by the PILh, PPLh, NACWO, Home Office Liaison Contact (HOLC) and establishment licence holder. Any issues and incidents that PILh need to be aware of are discussed at this meeting. Further to this, this platform is used to disseminate information from Animals in Science Regulation Unit (ASRU), National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and other organisations and to provide details of training opportunities to enhance welfare and research practice. We have also embarked upon a strategy of ensuring that PPLh and PILh undergo regular refresher training at least every 3 years to ensure that they remain abreast of changes to best practices and aware of their responsibilities under the Animals (Scientific Procedures) Act 1986 (ASPA). These advances are further communicated through our active team of NACWOs and regular email updates. We will also stay up to date with specific cancer groups, databases and alternatives (NC3Rs) for cancer models such as https://resources.researchanimaltraining.com/faqs/breast-cancer-research-alternatives-database; https://data.jrc.ec.europa.eu/dataset/352f7dfd-05cf-434b-a96a-7e270dc76573.

170. Development, breeding, maintenance, and validation of genetically altered rodents

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Genetically Altered Animal, Knockout Transgenic, Knockin Transgenic, Genome Manipulation, Embryo Transfer

Animal types	Life stages
Mice	adult, pregnant, juvenile, embryo, neonate, aged
Rats	juvenile, adult, pregnant, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is two-fold:

- to create, breed, validate and maintain colonies of rodents with genetic modifications (changes to their genes)
- use cryopreservation (controlled freezing) techniques to archive and preserve the integrity of the genetically modified rodents for future use.

The intention is to supply research teams within the company or external collaborative research establishments, with genetically altered animal (GAA) models that are relevant to the human disease condition to support research into developing understanding of disease and treatments for 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?

GAA models are vital to further the understanding of how genes work in human health and disease (Chenouard V *et al* 2021). The ability to alter genes within a living organism is a powerful tool to directly investigate the importance of a gene and / or it's protein products in specific biological/biochemical pathways and further the understanding of its function in disease states. Characterisation (in depth study) of such genetically altered (GA) rodents may lead to the development of new animal disease models displaying some or similar characteristics to those seen in human disease conditions. These new animal models may then be used to improve the understanding of the origins of human disease and in the development and evaluation of potential new medicines which may ultimately result in a cure or treatment to the disease condition in humans.

It is important for the company to leverage GAA technology to:

- generate rodent models to discover the function of genes (and/or their protein products) in the underlying biology associated with various human disease states
- establish translational (human disease-relevant) models for pre-clinical research that better reflect the human disease condition
- provide an essential resource for understanding disease mechanisms & biomarkers, improving diagnostic strategies and for testing therapeutic interventions
- support the company's emerging focus on using genetic & genomic technologies to identify and prosecute novel targets to develop next generation medicines

What outputs do you think you will see at the end of this project?

- Creation, re-derivation, validation, breeding and supply of novel, and existing GA rodent strains for scientific advancement
- A well validated, robust and reliable platform for generating high quality genetically altered rodents, through continual refinement of both genetic manipulation techniques and in vivo procedures
- Preservation of genetically altered lines of animals for future use, through established cryopreservation (controlled freezing) techniques reducing animal usage and avoiding extended breeding programs
- Publications or the sharing of new information subject to intellectual property restrictions

Who or what will benefit from these outputs, and how?

Genetically altered rodents have made significant contributions to human biological and medical research through supporting the development and testing of novel therapies, treatments and cures for diseases and disorders. GA models produced under the previous

project licence had an impact across a number of disease areas (including:- Neuroscience, cardiovascular, immunological, respiratory and oncology). For some examples see:

- Lomas DA et al; Development of a small molecule that corrects misfolding and increases secretion of Z α1-antitrypsin. EMBO Mol Med (2021)

- Vantourout J C et al; In Vivo Half-Life Extension of BMP1/TLL Metalloproteinase Inhibitors Using Small-Molecule Human Serum Albumin Binders. Bioconjugate Chem. 2021, 32, 2, 279–289.

Benefits from the output of this project licence are expected to be:

In the short to medium term, scientific projects will benefit from the use of specifically requested GA rodents (through this project licence) to enable scientific studies, generate meaningful data and push projects forward towards the generation of novel therapeutics.

An additional medium term key benefit will be delivered through assisting other project licensees in meeting their objectives (discovery and development of new medicines) with supply of scientifically validated GA models, delivering the most appropriate animals for the task.

Ultimately, in the long term, the aim is that patients suffering from disease and disabilities will benefit from any new therapies, cures and treatments that may be attained through supporting data generated from GA rodents provided under this project licence.

How will you look to maximise the outputs of this work?

Data generated by research teams using GA rodents provided under this project licence are 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), whereby it does not contain information that is subject to intellectual property constraints, it will be published as appropriate. The company also supports the view that publication of unsuccessful approaches ('negative data') is a valuable scientific output from properly conducted research, and these types of data would not be excluded from a publication strategy. Where possible, GAA models will be shared with academic institutes to directly support scientific research and potentially reduce overall animal usage.

Information generated using this licence is stored in a searchable, secure company database, so that it is always accessible to other internal company researchers. Therefore, data will be accessible in the future, even after likely project and personnel changes, and the information will be a valuable future resource when looking to reproduce or regenerate certain GA rodent lines.

As part of the GA rodent platform strategy outlined in this project licence, we will look to collaborate with both internal and external partners that are experts in the fields of bioinformatics, designing genome editing tools and GA model characterisation. This will allow generation of reagents, genetic characterisation and genotyping to be done externally in most cases, freeing up resource internally that can be used elsewhere on this platform.

Species and numbers of animals expected to be used

- Mice: 40000
- Rats: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents, especially mice are an ideal species for use when creating genetically altered models for research purposes due to their ability to be robustly genetically manipulated and their similarities with humans in term of physiology and genome (the complete set of genes present in an organism).The technology employed to generate genetically altered mice is well-developed, and such animals are widely used across the medical, academic and scientific research communities. The multitude of successful genetically altered models available proves they are a suitable and robust species for gene manipulation. Additionally, there is the advantage of having a fast reproductive cycle lending itself to the high throughput nature of research and to generating and managing breeding colonies to support this. All life stages will be used throughout protocol 3 on this project licence as the nature of breeding and maintaining colonies of genetically altered mice affects the full life cycle. Juvenile, young adult and, on occasion, adult animals (up to 1 year of age) will be used throughout protocols 1 and 2 of this project licence as creation of GA rodents requires the harvest of oocytes from, or implantation of embryos into,
females with sexually mature reproductive systems in order to bring pups to term and raise them until weaning. Aged animals (up to 24 months of age) will be used on protocol 4 of this project licence as using older animals is essential for the development of certain types of model looking into diseases related to natural aging, for example age-related neurodegeneration.

Typically, what will be done to an animal used in your project?

Typically animals on this project licence will remain on the same protocol for the duration of their use unless being transferred to protocol 1 (for superovulation), protocol 2 (wild type females only for embryo transfer), protocol 4 (if aged animals are required) or authority of another project licence for scientific use. See below for what will typically be done to an animal on each protocol.

Protocol 1 - Superovulation

(a) Administration of agents (such as hormones) by intra-peritoneal or subcutaneous injection (typically 2) to increase the yield, and stimulate the release of, premature egg cells (superovulation). If early stage embryos are needed these animals may also be mated following superovulation.

(b) Humanely killed following successful mating, and egg cells / embryos harvested post mortem for cryopreservation, in vitro fertilisation or subsequent implantation into recipient females (performed under protocol 2). The animals themselves will not progress on to any other protocols.

(c) Superovulated female mice that have not successfully mated, may be used for natural mating if required

Protocol 2 - Embryo Transfer

- Wild type (non-genetically altered) female mice will be rendered pseudopregnant (false pregnancy) by mating with a sterile male, these females are referred to as recipients.

- Surgery will be performed to transfer early stage embryos (generated by either superovulation (protocol 1), in vitro fertilisation, revival of cryopreserved embryos or natural mating) into the reproductive tract of recipient pseudo-pregnant females.

- Surgery will be done under general anaesthesia, sections of the reproductive tract will be exposed for embryo delivery. Incisions will be stapled / sutured and the animals allowed to recover.

- Pre and post-surgery, recipient females will receive analgesics (pain relief) and may receive antibiotics as agreed in advance with the named veterinary surgeon (NVS), usually when the animal is under general anaesthesia to minimise discomfort.

- Where appropriate (i.e. where later stage embryos (blastocysts) can be used), the embryo transfer will be carried out using non-surgical methods, and in such cases the pseudopregnant female mouse may be immobilised using brief anaesthesia. The expectant mothers will be bought to term (typically 21 days post-surgery) with the offspring produced being authorised by protocol 3 from two thirds of the way through gestation. After weaning of offspring (typically at 21 days of age), dams will be humanely killed and will not progress on to any other protocols.

Protocol 3 - Breeding, maintenance and validation of genetically altered animals (mild)

- Animals born through protocol 2 procedures will be ear notched at typically 2-4 weeks of age for identification purposes. The tissue removed during the identification process may also be used to genotype (genetically identify) the animals.

- If further information is needed to characterise the genetic modification (e.g. to determine gene expression levels, impact on biomarkers, changes in clinical pathology or histopathology) then animals may have a blood sample taken from a superficial vessel (typically once) for this purpose. If larger blood volumes are required or alternative tissues / methods for collection are needed for genetic modification characterisation purposes and where Schedule 1 methods have the potential to compromise the samples (e.g. blood clotting, contaminated cerebrospinal fluid, damaged or compromised tissue); these will be obtained as part of a terminal procedure under non-recovery anaesthesia.

- Typically, once genotyped, animals will be:

    - used for breeding to maintain the colony
    - or kept alive and used as sentinels (only wild type animals that have undergone no regulated procedures) within our animal facility (to reduce the need to order in extra animals for this purpose)
    - or transferred to protocol 1 (female mice only), protocol 2 (female WT mice only) or protocol 4 of this project licence
    - or transferred to another project licence within the company or externally to a collaborator for further scientific study

- At the end of use on this protocol animals will be humanely killed

Protocol 4 - Maintenance and validation of genetically altered animals up to 24 months of age (moderate)

- Any GA rodents (and their wild type counterparts), that are required to be aged up to 24 months of age will be transferred to protocol 4; and may be transferred to protocol 4 from weaning. In most cases animals moved on to protocol 4 would have already been genotyped and genetically characterised, if not this will be done on protocol 4. Typically animals will have the same experience as those on protocol 3 above. Aged animals will not be used for mating and will not be kept alive and used as sentinels.

What are the expected impacts and/or adverse effects for the animals during your project?

Protocol 1 - Superovulation

- The superovulation procedures are expected to result in no more than transient discomfort, as a result of the physical insertion of a needle either intra-peritoneally or subcutaneously.

Protocol 2 - Embryo Transfer Surgery

- Animals may experience discomfort moving around for up to 24 hours post-surgery, but a comprehensive peri-operative pain management regimen will be in place to keep pain and suffering to a minimum.

- Animals are expected to display abnormal behaviour immediately following surgery as a result of the anaesthetic (e.g. subdued, "wobbly" gait, lack of appetite). These effects will be transient, lasting no more than 60 mins following surgery as the anaesthetic wears off. Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within 2 hours.

- Less than 5% of animals may show local effects such as skin inflammation, infection or wound breakdown.

Protocol 3 - Breeding, maintenance and validation of genetically altered animals (mild)

- Ear notching (removal of a small amount of tissue (1-2mm) from the ear) may cause very mild transient localised discomfort

- Removal of blood from a superficial vessel may cause mild transient localised pain.
- Incidence of adverse effects are expected to be low, occurrences of pregnancy complications are monitored and if above background frequency for a particular strain, will be investigated with the NVS, project licence holder and named animal and welfare officer (NACWO).

Protocol 4 - Maintenance and validation of genetically altered animals up to 2 years of age (moderate)

- Ear notching (removal of a small amount of tissue (1-2mm) from the ear) may cause

very mild transient localised pain but unlikely in most cases.

- Removal of blood from a superficial vessel may cause mild transient localised pain.

- Age-related adverse effects may be seen in line with genetic background, including but not limited to, skin conditions, abnormal activity, weight fluctuations, eye complications and tumours.

- Some GA strains may have the potential to develop a harmful phenotype after a certain age in addition to the normal age-related adverse effects expected in line with their genetic background. In such cases if a harmful phenotype is expected for a particular GA strain, prior to these animals being granted authority on this licence, protocol 4 will be amended to include a list of the additional likely adverse effects, refinement controls and humane endpoints keeping impact on animal welfare to a minimum. In all cases animals will be killed before reaching that age and before the onset of clinical signs, unless such clinical signs are inseparable from the process of maintaining animals to a required age (up to 24 months) for scientific study, or unless animals are moved on to another project licence for a specific purpose (continued use).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 1 - The expected severity is mild and 100% of animals are expected to experience this

Protocol 2 - The expected severity for animals undergoing surgery (majority of animals on this protocol) is moderate and 100% of these animals are expected to experience up to this severity. The expected severity of animals undergoing non-surgical embryo transfer (this technique will not typically be used, unless embryo stage required is compatible with technique, so will only be a minority of animals on this protocol) is mild and 100% of these animals are expected to experience up to this severity.

Protocol 3 - The severity classification of this protocol is mild, however the majority of animals (~80%) on this protocol are expected to to experience a severity of no more than sub threshold

Protocol 4 - The severity classification of the protocol is moderate, however the majority (>90%) of animals on this protocol are expected to experience no more than mild severity

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A wide range of information from in silico (computer modelling) or isolated cell systems is available to increase our understanding of how potential targets for medicine may function or how they can be affected by treatment. However, due to the need to understand how different cell types and physiological processes work together and affect each other to influence biological mechanisms, relevant data cannot always be generated using such isolated cells in artificial plastic (in vitro) setups. As such, investigating diseases of complex systems such as the cardiovascular, respiratory, immune, gastrointestinal and central nervous systems, require the use of whole functioning tissue that can be found within living animals.
Understanding the response of a potential treatment in a whole animal with a physiology that is common with humans (enabling scientists to take into account complexities such as how the potential medicine is distributed and metabolised throughout the body) is vital to guide progression of such potential medicines to human clinical trials.

For these reasons it is necessary that genetically altered animals are produced, validated & maintained under this licence and made available to researchers so that specific traits (characteristics) of certain genes implicated in disease can be studied in complex living systems. Many of the effects of genetic changes and biological processes under investigation involve complex pathways that cannot be replicated outside living animals (ex-vivo).

The GA rodents created, validated & maintained under this project licence will support research efforts across a multitude of disease areas some of which require intact behavioural responses that only a conscious living animal can provide. Non-animal methods are not always able to model or replicate the complete array of behavioural responses or indeed the cellular, molecular and physiological interactions required to fully understand how genetic alterations result in normal (healthy) or abnormal (diseased) processes.

Which non-animal alternatives did you consider for use in this project?

Project teams requesting new GA models under this licence will work with an independent scientific review committee to understand the feasibility of using animal alternatives prior to reaching any conclusion to progress with in vivo work. This will be discussed with the project licence holder and often such discussions will involve, alternatives such as the use of organoid preparations & organ-on- chip technologies, ex-vivo and in vitro tissue and cell culture platforms and human cell-based assays. If any of these can address the same decision making scientific end points that the requesting project team requires from a genetically altered rodent model, then these will be pursued as alternatives. Any viable non-animal alternative options discovered may be used as examples to challenge requests coming in to this project licence from other research teams.

Why were they not suitable?

Often the inability to robustly generate data in a non-animal environment will be the main factor in a research team pursuing the development of a GAA rodent model. Specific issues often arise in that cellular or tissue based platform technologies have a limited number of cellular interactions and do not provide the full level of complexity and integration available within a living animal i.e. the inter- connected body-wide systems where cells and organs continuously communicate. Additionally these platforms 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. As such, these technologies (while continuing to play a vitally important and complimentary role to animal experiments) are not yet fully developed and characterised to a stage where we can have confidence in all applications of the clinical relevance of data generated from using them. They do not adequately model the complete array of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes. Under circumstances such as these, the animal model is critical and decision making in progressing therapeutics towards the clinic.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals to be used is based upon the numbers of animals used across previous GAA-focused licence authorities previously held within the company. These numbers take into account historical requests for GA rodent models over the last five years as well as current and future internal demands for such models. There is an anticipated steady rate of projects likely to require information provided through GA rodent models that cannot be gained from non-animal alternatives. Also considered in these estimates is the creation of novel GA rodent models, and the likely demand thereof, something not performed under the previous GAA project licence, and likely something that is to be used throughout the lifespan of this project licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure the unnecessary production of GA rodents is avoided, available databases will be searched to identify existing models that could be obtained. Examples of resources available include:

- NC3Rs mouse database: http://www.nc3rs.org.uk/
- Mouse Genome Informatics: http://www.informatics.jax.org/ PubMed: http://www.ncbi.nlm.nih.gov/
- Jackson laboratory: http://jaxmice.jax.org/index.html

Animal numbers for experiments are assessed by sex, age, genotype, phenotypic traits (physical characteristics) and statistical relevance to the experimental needs and these are all considered prior to onset of the breeding programme to produce animals for study. Animals will only be bred to deliver an investigators requirement that has been established using robust statistical methods.

This licence will adopt the most efficient methods for genetically manipulating harvested embryos (such as electroporation) and in combination with more refined superovulation techniques (being investigated to increase the number of eggs produced per female) will

translate through to a decrease in the number of donor females used as greater yields of higher quality, viable embryos will be generated from each female.

When creating GA mice in house, data will be captured from each transfection session, taking into account starting numbers of embryos and tracking this to numbers of viable embryos for implantation, through to live pups born following embryo transfer. This will allow us to optimise the number of embryos to transfer to each recipient female and ultimately optimise the number of pseudopregnant females we need to reach the required number of GA founder offspring.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Good colony management and regular communication with requestors are key to minimising wastage within the breeding facility. To ensure these colonies are breeding efficiently we monitor breeding performance related to the expected background strain information and Mendelian ratio (the likely occurrence of differing genotypes (genetic variants)) anticipated from the genetic modification under study. Careful breeding programmes will be put in place to maintain the minimum numbers of animals in order to deliver the animals of relevant genotype. These breeding plans are subject to regular review by the project licence holder, responsible technician and technology manager to optimally meet anticipated demand. Wild type animals bred unavoidably as a consequence of a GA breeding programme, that have had no regulated procedure performed on them, will be made available for use on other scientific projects within the company (e.g. tissue donation, sentinels for health monitoring).

This licence provides a cryopreservation service (preserving biological cells/tissues/organs by cooling to very low temperatures) of genetically altered lines of animals for research partners, allowing the long-term storage of important lines that are not required at that moment but may be in the future. Additionally lines are monitored for usage and routinely cryopreserved to ensure they are only active when studies have been assigned to them. This enables there to be a reduction of animals that are maintained in breeding colonies within animal facilities. Where possible sperm cryopreservation will be used as this significantly reduces the number of animals required to freeze down sufficient stocks (compared to cryopreserving from females). Embryos will be cryopreserved if there is a need to preserve the genomes of both parents (for example an inbred line or strain on a complex background).

The background genetic strain used for generating a new model will be carefully considered to avoid producing unwanted mice or impacting the scientific output of the study they are being supplied for. Scientific techniques (e.g. single-nucleotide polymorphism analysis) will be employed where needed to confirm genetic background makeup and reduce the number of breeds required to to transfer an introduced genetic modification from one background strain to another (e.g. Balb/c to C57BL/6J genetic backgrounds). Additionally animal numbers will be optimised by effective liaison and rigorous review with project teams to ensure strains of the most appropriate desired specification are always used (the best model for the disease area).

Numbers are also optimised where several research programmes may use the same animal model (or indeed tissues from the same animal) and where required external collaborators also.

Newer superovulation methods (developed by the Division of Reproductive Engineering in the Centre for Animal Resources and Development at Kumamoto University) employ the use of goat inhibin antiserum in the so call "hyperovulation" process to increase the yield of eggs from a single female compared to standard superovulation. The advantages of this will be a potential reduction in the number of donor females used, as more eggs / embryos can be obtained from fewer animals. When implementing new techniques in this area, the quality of the eggs produced and the success rate of pregnancy and live pup birth rate will be carefully monitored before completely adopting new methods of superovulation.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The methods that will be employed in this project licence are as follows:

Superovulation techniques - Hormones are administered to WT or GA females typically by two intra-peritoneal or sub-cutaneous injections, given at a time & dose driven by the characteristics of the background rodent strain of choice. This is the least invasive and most effective way of encouraging females to ovulate and copulate while increasing yields of oocytes or embryos compared to what would normally be obtained through natural oestrus or mating. Mice that have undergone this procedure will be killed by a schedule 1 method prior to harvest of oocytes or embryos. There is a wealth of data from past internal project licences using this technique that indicates no adverse effects, in addition to the mild discomfort caused by the injections, result from this superovulation regimen.

Induction of pseudopregnancy - Recipient females of the donated embryos, will be induced into a pseudopregnant state through mating with a sterile male prior to embryo transfer. There is a very low risk of injury to the females caused by rough treatment from males. Fighting/injuries will be reduced by ensuring, where possible, that females are in oestrus, and by replacing over vigorous males. Other methods for inducing pseudopregnancy such as repeated stimulation of the cervico-vaginal region with probes or swabs, or administration of substances intra-peritoneally, sub-cutaneously and /or into the vagina do exist. However these methods involve levels of restraint or mild pain from injections, and compared to natural mating with sterile males (held under protocol 3 of this licence), are less efficient, more invasive and more stressful to the female.

Surgical embryo transfer - The most well established and robust method for transferring embryos to recipient females is through direct surgical implantation to the reproductive tract under recovery anaesthesia. The surgical methods employed in this licence will be minimally impactful as there is a large amount of expertise available in performing these surgeries making them as short in duration as possible. Additionally the methods employed will keep pain, suffering, distress and lasting harm to a minimum through use of carefully considered analgesic and anaesthetic regimens as well as optimised recovery and post op monitoring processes.

Non Surgical embryo transfer - Where later stage embryos for implantation can be used (e.g. blastocyst stage), non-surgical embryo transfer techniques will be employed. This technique involves the use of a trans cervical pipette (a device that is inserted through the cervix to expel embryos into the uterus) and has advantages over surgical embryo transfer methods in that no invasive surgical procedures are needed and thus has a lower severity. This technique may use brief general anaesthesia for restraint.

The use of genetically sterile males- Genetically sterile male mice (e.g. Protamine 1 (PRM-1) KO mice) held on protocol 3 of this project licence, will be used to render recipient mothers pseudopregnant prior to undergoing embryo transfer. This method surpasses the previous method of using vasectomised males, as the animals are born sterile and therefore will not have to undergo general anaesthesia and invasive surgical procedures to render them sterile.

Non-aversive handling techniques - the use of cupping and / or tunnel handling techniques to retrieve animals will be encouraged and employed where possible as these methods have been shown to be less aversive to the animals compared to e.g. handling by the base of the tail. The use of non-restrictive restrainers will also be used where possible to minimise stress and discomfort for the animals.

The methods 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 producing GA rodent models.

Why can’t you use animals that are less sentient?

The end users of this licence (those researchers and project teams that are requesting GA rodent models) require gene modifications to be made in species that have fully-functioning, biological mammalian systems that are comparable to humans (e.g. cardiovascular, respiratory, immune, gastrointestinal and central nervous systems). Rodents, such as mice, are the least sentient and most feasible to use species that offer such comparisons to humans. This allows researchers to determine the effects of specific genetic manipulations, in the presence and absence of potential medicines, and the complex interactions these have with relevant physiological, fully-functioning mammalian systems.

It is not feasible to use more immature life stages as creation of GA rodents requires the implantation of embryos into females with mature reproductive systems in order to bring pups to term and raise them until weaning.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Embryo Transfer Surgery - Working closely with experts including the NACWO and NVS, the embryo transfer surgery technique will be refined through optimising various aspects of the procedure. For example, this may be through, but not limited to, adopting increased post op monitoring, core body temperature monitoring throughout procedure, alternative wound closure techniques, enhanced anaesthesia setup (to improve ergonomics and incorporate the use of face masks when clipping fur) and administration of additional perioperative analgesia.

Non-surgical Embryo Transfer Surgery (NSET) - The embryo transfer technique (performed solely under protocol 2) is via a surgical laparotomy method, and only applicable to a small number of animals used on this licence (pseudopregnant embryo recipient females). This is due to the need to use early (1 to 2-cell stage) embryos for in vitro fertilisation (IVF) or genetic manipulation using electroporation. In order to maintain viability, these early stage embryos need to be transplanted into recipient female oviducts with minimal in vitro culture, this is only possible via a surgical procedure for this stage of embryo. NSET involves implanting embryos further down the reproductive tract into the uterine horn, entering through the vagina using a trans cervical pipette. Therefore, only later stage embryos are suitable for this technique (e.g. blastocyst stage). Currently culturing on from earlier stage embryos to blastocyst stage in vitro, leads to significantly reduced viability and in many cases loss of the embryos. Microfluidic embryo culture devices have made steps in the right direction to improving embryo development in vitro (Mancini V et al 2021), however these still remain to be fully validated in the field and are not readily available commercially. If and when NSET techniques become reliable and viable to use in the lifetime of this project they will be investigated with the intention of reducing the number of animals having to go through surgical procedures for embryo transfer.

Genetic identification - Where possible non-invasive methods (compared to biopsy) will be sought to genotype (genetically identify) GAAs, for example by coat colour or observable reporter gene such as green fluorescence protein.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Diehl KH et al (2001) - A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes: JOURNAL OF APPLIED TOXICOLOGY 21, 15–23

Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).

Kilkenny C et al (2010) - Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6).

LASA (2017) - Guiding Principles for Preparing for and Undertaking Aseptic Surgery

LASA (2015) - Guiding principles on good practice for animal welfare and ethical review bodies.
Naomi Nakagata (2016) - Reproductive Engineering Techniques in Mice Technical Manual.

Centre for Animal Resources & Development, Kumamoto University, Japan

NC3R's (2019) - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies

Percie du Sert N et al (2019) - The ARRIVE guidelines 2019: updated guidelines for reporting animal research. BioRxiv. 2019: 703181.

Prescott MJ, Lidster K (2017) - Improving the quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156

Review of harm-benefit analysis in the use of animals in research (2017) - Report of the Animals in Science Committee Harm-Benefit Analysis Sub-Group chaired by Professor Gail Davies

Smith A et al (2018) - PREPARE: guidelines for planning animal research and testing. Lab Anim; 52(2):135-141.

Wilkinson M J A et al (2020) - Progressing the care, husbandry and management of ageing mice used in scientific studies. Lab Anim; 54(3) 225–238.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the 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 the company 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) of which I am a member. I am also separately aware of the 3Rs 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 the company centrally and effectively via these forums. Additionally I also plan to attend external conferences, meetings and training courses as well as collaborate with other establishments to learn and share best practices in the field of genetically altered animal research.

171. Provision of an outsourced drug discovery platform for metabolic and cardiovascular diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Metabolism, Cardiovascular

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop new and improved treatments for metabolic and cardiovascular disease. As part of this work we will optimise experimental models of disease to ensure they are fit for purpose.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 crucial data in the preclinical (before human dosing) development of potential new drugs, generating important information that cannot be found without the use of animals. The clients that we work with are developing test agents (potential new drugs) which will hopefully contribute to helping people with various metabolic and cardiovascular diseases where current treatments are not available or need

improving upon. For example, Pulmonary Arterial Hypertension (PAH) is a rare but severe cardiovascular disease where 40% of patients die within 3 years of diagnosis. To be able to test new drugs for diseases like this, it is necessary to use rodent 'models' which mimic areas of the disease to provide proof-of-concept data and to determine which drugs are likely to work well in humans as a treatment for the disease.

What outputs do you think you will see at the end of this project?

This project will generate important data in the preclinical development of potential new drugs for metabolic and cardiovascular disease. We aim to use this licence to test potential therapeutics in each of the disease areas listed, and to progress the most promising treatments which reduce disease severity through various stages of drug development and into the clinic. We are currently working on a series of drugs for PAH which have shown promising results in our rats that have a form of PAH. We aim to support the progression of these drugs through to the stage that they are able to enter clinical trials within the duration of this licence. We also aim to work with several other clients in each of these disease areas to offer a similar service.

These studies will determine which test agents are suitable for further preclinical development and for progression to clinical trials. They will also identify test agents which are not suitable for further development. This is an important benefit, as it will streamline research and ensure that animals are not used unnecessarily in developing compounds that will ultimately fail further down the development pathway. We prioritise ensuring that our models are designed such that unsuitable test agents are identified as early as possible in the testing process. One particular example of this is in our PAH work, where a class of new drugs are known to have the potential to cause an unwanted side effect of heterotopic ossification (bone formation in inappropriate locations). This project includes a protocol to allow us to rule out test agents with this side effect early in the development process.

Data from studies performed in this project may also be used in applications by our clients for further funding or as part of applications to allow test agents to be progressed into clinical trials.

Who or what will benefit from these outputs, and how?

This project will generate important data in the development of potential new drugs. Work under this licence is expected to result in the progression of new treatments for cardiovascular and metabolic disease through the stages of drug development and ultimately into human clinical trials.

Our focus on using a science-led approach will enable key decisions to be made at each development stage on whether a test agent is likely to become a successful drug. This allows test agents which are not suitable as drugs to be abandoned at an early stage using the fewest number of animals possible for each test agent. The identification of test agents as unsuitable for use in humans at an early stage of development will also ensure a better success rate in the drug discovery process than has been seen previously in the pharmaceutical industry.
Ultimately, this project will contribute to the successful development of new drugs for cardiovascular and metabolic diseases, which will benefit patients who currently have limited treatment options available.

PAH is a disease with an estimate global prevalence of 1%. This disease can affect both

sexes and all age groups, with a higher risk in females. PAH has a high mortality rate, with patients surviving on average only 3-5 years from diagnosis. Effective treatments would therefore have a significant impact on the lives of patients diagnosed with PAH.

Non-alcoholic fatty liver disease (NAFLD) has a global prevalence of 25%, and it is estimated that up to 5% of the UK population may be affected by the more serious form of the disease, non-alcoholic steatohepatitis (NASH). These diseases are closely associated with obesity, and therefore the number of patients suffering from them and who would benefit from new treatments is likely to increase. The average age of people with NASH is 40-50, with this age decreasing due to the increasing problems with obesity in society. The mortality rate is higher for patients with NASH than the general population, with liver damage from NASH increasing risk of liver failure and cancer.

How will you look to maximise the outputs of this work?

All studies are designed such that the outputs from each animal are maximised. Expert knowledge is utilised not only from within the preclinical (animal work) team performing the animal studies, but also from other teams at our company and our clients' companies. This ensures that all relevant work that has been performed in the laboratory is taken into consideration when designing the animal studies. The *in vitro* (in the dish/test tube) and bioanalysis teams at our company are experts at analysing tissue and blood samples collected from animals, and they help with details of sample collection and storage to ensure that the samples are collected and stored in the best way possible. They are also experts at working with small samples, particularly very low volume blood samples, meaning that they can analyse lots of different biomarkers and test agent levels from each animal.

In addition, we will seek expertise from our established networks both within our establishment and further afield, to ensure that we make use of any new knowledge or better methods of performing animal studies. We will also use these networks to give information to others about any ways in which we can help via our research. This will include sharing information about unsuccessful approaches in addition to sharing information on any refinements and improvements. We will maintain good communication with managers of the animal facilities to ensure that any tissues from animals being killed that are not required for our work can be made available to other researchers.

Due to the nature of the work undertaken in this project we are unlikely to be able to publish data, as this would put ourselves or our clients at a competitive disadvantage. However, where advances are made in study design, we will publish or share these wherever possible.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Both mice and rats are well established ‘models’ for use in both preclinical drug research and more generally in research into metabolic and cardiovascular diseases. This is due to their similarities to humans, with these 'models' mimicking aspects of human physiology and disease. They are very well characterised, meaning that there is a lot known about how the body works in mice and rats, both when healthy and with diseases. Adult rodents will be used for the work outlined in this project as we wish the biology of the animals to be fully developed to better represent the patients of the diseases the test agents are designed to treat.

Typically, what will be done to an animal used in your project?

The majority of animals used in this project will be part of studies that aim to test whether new drugs can improve or prevent development of symptoms associated with metabolic or cardiovascular disease. This will include investigation of any potential unwanted side effects that could be caused by the new drugs. Drugs will most commonly be administered intraperitoneally (into the abdominal body cavity), subcutaneously (under the skin), orally (either using a dosing tube or via adding the test agent to the food or drinking water) and less frequently intravenously (into a vein) or intramuscularly (into the muscle). On occasions a slow-release device will be implanted for the subcutaneous or intraperitoneal administration of drugs. At the end of all studies blood and/or tissues will be collected. These may be frozen or preserved using a fixative. Tissue may be placed into fixative after death or the fixative may be injected into the bloodstream or directly into the heart of an anaesthetised animal who will not regain consciousness between the injection of fixative and death.

In metabolic studies investigating NASH (non-alcoholic steatohepatitis) and NAFLD (non-alcoholic fatty liver disease), mice will be fed a high fat (or normal) diet to cause them to develop the liver problems associated with the disease. In these diseases, fat builds up in the liver, accompanied by liver inflammation in the case of NASH. Long-term, this causes damage to the liver and can reduce liver function. Animals will usually be fed the altered diet for 4 or more weeks and be administered with test agent throughout the study. Blood samples may be taken during the study to monitor the disease progression and effectiveness of the drug administration. At the end of the study terminal blood samples will usually be collected from anaesthetised animals immediately before death (meaning the animals are unconscious prior to blood collection and will not re-awaken) and mice humanely killed prior to collection of tissues.

Our PAH studies will use one of two methods to induce the disease in the animals. In the first, the sugen-hypoxia (SUHx) model, the majority of animals will be subjected to a hypoxic environment of 10% oxygen for 3-4 weeks (the equivalent of being at the top of a high mountain). A small subset of control animals will remain in a normoxic (normal air) environment throughout the studies. Animals may either be humanely killed immediately upon removal from the hypoxic environment or returned to a normoxic environment for several weeks prior to killing. Animals will be administered with a PAH enhancer by subcutaneous injection immediately prior to entering the hypoxic environment, and this administration may be repeated during the hypoxic period. They will also be administered with test agent at regular intervals. This will usually be during the hypoxic period in mice and after the hypoxic period during a period in normoxia in rats. Blood samples may be taken during the study to monitor progression. At the end of the study, the heart and blood vessels may be cannulated to measure pressure, prior to collection of a terminal blood sample. Cannulation is performed in terminally anesthetised animals, and involves the insertion of a thin, flexible probe into the heart via a connecting blood vessel. Animals will

be humanely killed prior to collection of tissues. The second method is the monocrotaline (MCT) method. This method will only be performed on rats, who will be injected with MCT via subcutaneous or intraperitoneal injection to induce a form of PAH disease which is similar to human PAH. The MCT only needs to be administered on one occasion to induce disease which is similar to human PAH. A small subset of control animals will be administered with vehicle (the liquid the drug is administered in but without drug). The animals will then be administered with test agent at regular intervals, with sample collection and heart cannulation as for the SUHx method.

In heterotopic ossification (HO) studies investigating the potential unwanted side effect of some test agents designed to treat PAH, animals will be subjected to muscle injury either via chemical or physical means. The reason for the muscle injury is that HO normally only occurs at the site of an injury, not in healthy tissue. Therefore, an injury must be caused to check if this side effect would be a problem if the test agent was used in humans. Muscle injury will be performed under anaesthesia and this will involve either injection of a toxin into the muscle or physical muscle damage by the dropping of a ball bearing onto the muscle. The injury is made to one of the rear limbs. Animals will be administered with pain relief if needed. However, this is not usually required, with animals able to move normally after the injury. Animals will be administered with test agent on one or more occasions, often directly into the site of muscle injury. Blood samples may be taken during the study to monitor progression. At the end of a study a terminal blood sample may be collected immediately before death and all animals will be humanely killed prior to collection of tissues.

Where possible, we will make any tissues not required for our work available to other researchers after the death of the animal.

What are the expected impacts and/or adverse effects for the animals during your project?

In studies investigating NASH/NAFLD mice will be fed a diet high in fat and/or given sugars in their drinking water. This may cause obesity due to the high fat content of the diets used. However, where the diet is less palatable (meaning that the animals do not like the taste as much as normal), animals may lose (or not gain) weight meaning that their weight will be lower than that of control animals at the end of the study. Diets will be made as appealing as possible to the animals, but sometimes the use of a less palatable diet can give better results in the studies and shorten the studies which reduces the overall impact on the animal. The fat in the diet may cause their coat to become greasy, which ultimately may lead to hair loss through over grooming.

During HO studies, animals may experience some pain associated with the induction of muscle injury. This will resolve within a few days of muscle injury occurring. In our experience, pain relief is not normally required; animals are able to move normally immediately after injury.

During PAH studies using the SUHx method, animals lose up to 10% of their body weight within the first 1-2 days of exposure to the hypoxic environment and demonstrate reduced activity. After this acclimatisation period, activity levels increase and eating behaviour and weight gain proceed normally. Animals who receive a PAH enhancer and exposure to the hypoxic environment and are then returned to the normoxic environment will develop a more severe disease. It is expected that some animals will be found dead in the rat model of this design. These animals suffer sudden death due to heart failure, and do not experience severe suffering. During PAH studies using the MCT method, rats similarly

develop features of PAH as in the SUHx model and may also experience sudden death due to heart failure.

The test agents administered in all studies in the project are not expected to cause any lasting harm. Animals may lose some body weight initially whilst adjusting to them. Where a slow-release device is used, these animals will undergo surgery under general anaesthesia, with quick recovery expected. The location of the slow-release device is not expected to have any effect on the animal's ability to move freely. Blood sampling is not expected to cause any lasting discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse - Mild: 38% Mouse - Moderate: 62% Rat - Mild: 14%
Rat - Moderate: 86%

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 work undertaken under this project licence cannot be fully replaced with *in vitro* (conducted in a laboratory without use of animals) or *ex vivo* (conducted on animal tissue but not in live animal) models. Wherever possible, work is performed in *in vitro* or *ex vivo* models prior to *in vivo* (animal) studies, to ensure that the *in vivo* studies are well designed and that the maximum amount of data is obtained from each study. This can be validating the target of a test agent and/or testing the action of the test agent itself in a suitable cell line to ensure the test agent is acting as expected before moving into animal studies. This may also be performed in *ex vivo* samples, providing data not available from established cell lines, but without requiring full *in vivo* experimentation. Our *in vitro* and *ex vivo* work ensure that wherever possible we generate proof of concept data before moving into animal studies, thereby reducing the number of animal studies performed in total and preventing test agents which are unlikely to be viable therapeutic agents being moved forward to *in vivo* studies.

However, isolated cells cannot reproduce the complex nature of the entire system of a living animal. This is particularly true for the areas of research covered by this licence. Metabolism involves complex interactions between different organs in the body, which cannot be recapitulated in the lab. Similarly, the cardiovascular system and flow of blood around the body cannot be recreated in a cell-based system.

Which non-animal alternatives did you consider for use in this project?

Work can be performed in the laboratory to ensure that test agents are acting as expected

before moving into animal studies. These may be using cell lines grown in the lab, or using tissues collected from animals. We have previously performed these types of experiments for example by looking at the effect of a test agent on liver cells collected from a mouse. We use these studies before moving into animals to make sure that test agents that are unlikely to be useful to humans do not get tested in animals.

Why were they not suitable?

Whilst cell-based work in the laboratory can be used to test how test agents act on cells, they cannot model the effect on whole organs, or the interaction between different organs which is a crucial element of metabolic and cardiovascular diseases. In addition, cell-based work does not test the effects the body might have on a test agent, for example how it gets into the blood, travels around the body and how it is removed from the body. No alternative is available that can replace the need to test potential therapeutics in a live animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used has been estimated by analysing the number of animals used on previous projects and looking at the number of animals required for each type of study. This was then combined with a prediction of likely demand of future projects to give the numbers in this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have extensive experience in designing experiments of the types in this project, which has given us confidence in the number of animals required to ensure that no animals are used unnecessarily, but also that the data generated is reliable. Through experience we tend to use a standard group size for some of the studies under this project, using 6 per group for heterotopic ossification studies and 14 per group for PAH studies. We regularly refer to the PREPARE and ARRIVE guidelines and make use of the NC3Rs Experimental Design Assistant to ensure that we are using the correct number of animals for every study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Data from pilot studies and previous experience are used to ensure that the numbers used are both as low as possible, but also large enough to generate reliable data. Within our company, a member of the wider team has generated a tool for performing power calculations and can be consulted to assist with study design. This expert regularly tests the tool against peer-reviewed power calculators and can adjust as necessary.

Where genetically altered animals are required, these will usually be provided by our breeding project, which will ensure that animals are bred efficiently using as few animals as possible by communicating need with colony managers. Where animals are obtained from external sources, only the number of animals required for the study will be purchased or imported.

Wherever possible, our *in vivo* scientists will be blinded to the treatment status of an animal, meaning that they do not know which treatment the animal is receiving. This reduces bias, enabling more reliable information to be gathered from a smaller number of animals. Where possible, those who carry out analysis on blood and tissues collected during the study are also blind to any treatment with test agent.

Baseline data (e.g. bodyweight, biomarker levels) are recorded and animals assigned to treatment groups to ensure there are no differences in baseline measurements between the groups at the start of the study. Once assigned to a treatment group number, the treatment status for each group will be assigned randomly.

Good planning ensures that within any series of studies we can control for variability that might be introduced. To limit this variability we look at using animals of a similar age/weight range, testing different batches of test agent in the lab first, using the same source of reagents (chemicals used during the experiments), keeping records of observations made and standardising as many components of an *in vivo* model as is practicable.

Whilst all studies in this project will require live animals, we will coordinate with other groups to offer post-mortem tissues for their work, where this is suitable for their work and does not affect the outputs from our own studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals will be housed in a purpose-built, state of the art facility, that is free of disease-causing organisms such as bacteria, viruses and parasites. They will have access to food, water and items that enhance their environment, such as tunnels, chew sticks and mezzanine levels to climb on. Our company staff and the animal care staff are competent in rodent welfare and will ensure that animal suffering is minimised. We aim to house rodents in groups to promote normal behaviour. However, aggressive behaviour can occasionally result in animals being singly housed to prevent injury.

We will use both mice and rats during this project. Some of these will have a genetic alteration that will not cause them harm, but which provides a more appropriate model in which to perform experiments using test agents for particular diseases.

Our NASH/NAFLD, HO and PAH studies are all performed using well-established

protocols, which reliably reproduce the important characteristics of the human diseases required for the testing of new drugs whilst minimising the harms to animals. We work to ensure that we introduce further refinements to our disease models wherever possible.

Test agents will often be tested first under our test agent profiling licence prior to testing in animals mimicking human disease. Where it is hard to predict the potential side effects of a test agent in the disease model, studies will be performed in a small number of animals first who will be monitored for at least 2 hours or overnight in the case of test agents where side effects are likely to take longer to be seen. Where side effects are observed, the dose may be altered to ensure no long-lasting side effects are seen before any longer-term studies are performed.

Dosing will always be performed using the least invasive route (e.g. oral gavage via food or water if possible), and where a needle is required using the smallest needle possible.

Amendment April 2022: For mouse PAH studies, the mice need to be under brief anaesthesia for an injection that requires them to stay still so that the drug doesn't touch the skin, potentially causing irritation. In most studies these injections need to be repeated several times. Therefore, under the guidance of the NVS we have seen some success in giving supportive subcutaneous saline to these mice to prevent dehydration and will continue to trial the use of it to gather more firm data on its effectiveness.

Why can't you use animals that are less sentient?

Adult rodents such as mice and rats are essential for studying complex diseases, where all systems must be similar enough to humans to find the most effective drugs. The complexity of metabolic and cardiovascular diseases requires the use of living animals, where the bodily systems are functioning and interacting with each other. Due to the length of the studies, which span weeks to months, terminal anaesthesia is not a possibility.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have a demonstratable history of refining our procedures and practices to minimise harm to our experimental animals. For example, we always use the smallest needle possible for dosing to minimise any pain and distress to the animal. We also investigate the suitability of test agents administered orally to be dosed via the drinking water rather than via oral dosing, where the dose is administered via insertion of a tube down the throat. We recently implemented this for a test agent, allowing us to switch from twice daily oral dosing to dosing via the drinking water. We will also sometimes use a slow-release device for dosing. Whilst this requires an initial surgical procedure, it reduces the need for the stress of regular injections.

During all studies, general animal condition and body weight will be monitored regularly. We are developing a detailed scoring system to monitor any side effects observed due to dosing. Animals will be housed in social groups in the vast majority of cases, with animals housed alone only where this is absolutely necessary (for example when an animal within a cage demonstrates aggressive behaviour). Where anaesthesia is used, care will be given to support animals during anaesthesia and recovery, using heat mats to maintain body temperature and providing soft diet and hydrogel to aid recovery as appropriate.

Where an altered diet is used, we will ensure that animals are introduced to the diet over a period of weaning, allowing them to acclimatise to the diet before a full switch is

performed. This will minimise the risk of weight loss due to a sudden switch to an altered and potentially less palatable diet. For new diets which have not previously been used we will review these using a small pilot study to ensure that they are palatable before using them for large numbers of animals. Where animals are hyperglycaemic (have increased blood sugar levels), we will replace the bedding more frequently when an increase in urination is observed.

For PAH studies, we have refined the method of injection of PAH enhancers that can cause sores at the site of injection by ensuring that we pinch the site of injection as the needle is withdrawn. This has reduced the incidence of sores. For mice, the PAH enhancers are administered under anaesthesia to further reduce the incidence of sores, with subcutaneous saline also administered to assist with recover from the anaesthesia given the potential reduce activity levels whilst in hypoxia.

For HO studies, the level of muscle injury has been refined to cause an injury that does not significantly affect the behaviour of the animals or reduce movement, but is still sufficient to assess the potential for test agents to cause HO.

We use microvettes (small capillary blood collection tubes coated with anticoagulant to prevent blood from clotting) to collect small blood samples from animals. This allows us to take small samples in the most efficient way possible.

Where possible, when animals are purchased from an external supplier these will be handled regularly during the acclimatisation period to reduce the stress for the animals when procedures commence.
Where diet consumption is not crucial to the outcome of a study, we also provide treats such as sunflower seeds after handling and procedures to assist in habituation for the animals to the procedures and any restraint required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be using PREPARE guidelines for the planning of studies and follow the latest version (2020) of the ARRIVE guidelines for ultimate reporting of data. The LASA document ‘Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals’ will be used when planning studies, to help guide study design. This document, particularly the section detailing the report of the Federation of European Laboratory Animal Science Association (FELASA) working group on pain and distress has also been used when setting the humane endpoints within this project. We use the resources on the NC3Rs website for guidance on the best practice for procedures such as blood sampling and for refinements to handling and husbandry. We will also regularly check the current list of LASA publications, to ensure that any relevant to work under this project are taken into consideration.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

When designing animal studies we consider the appropriate guidelines, including the guidance from the Laboratory Animal Science Association (LASA), the National Centre for the 3Rs (NC3Rs) and the Royal Society for the Prevention of Cruelty to Animals (RSPCA). This will include the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellent) and ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines which provide guidance on the design and reporting of

animal studies. This guidance will influence our study design. One example of this is the use of the NC3Rs guidance on the number and volume of bleeds to be taken from rodents in the design of pharmacokinetic studies. We regularly check the NC3Rs website, along with other external resources such as Norecopa (a resource of databases and guidelines from Norway) and Jax (a genetic animal strain resource providing a hub of research information), to ensure that we are using the most refined methods and are aware of any improvements to procedures that have been developed.

172. Provision of an outsourced drug discovery platform for the development of therapeutic drugs for cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, tumour models, therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is ultimately to develop new and improved treatments for cancer. We plan to develop new, or refine existing cancer models to improve the translatability of our work from animals to human medicine, resulting in the selection of the best drug candidates to take forward for further 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?

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020, with the number of new cases rising year on year (Ferlay J, et al. Global Cancer Observatory: Cancer Today.
Lyon: International Agency for Research on Cancer; 2020). In the UK, over a quarter of all

deaths involve cancer with the most common types being breast, lung, colorectal, prostate, skin and stomach cancer. In most cases, if cancer is left untreated it is highly likely that it will lead to death.

One cancer we hope to target is the particularly difficult to treat triple negative breast cancer (TNBC), which accounts for 15% of all breast cancers. There are currently fewer treatments available to treat this rare type of breast cancer, meaning that mortality rates are higher. Specific data on average age at death from triple negative breast cancer is not available, but with non-triple negative breast cancer mortality increases with age. In the UK in 2016-2018, almost half of deaths from breast cancer (48%) were in people aged 75 and over (Cancer Research UK website). However, younger women (<40 years old) are 1.53 times more likely to develop TNBC than older women (>60 years old), so age at death is likely to be lower in people with TNBC.

Whilst there have been significant advances in treatments for many cancers, deaths as a result of cancer still remain high. This is partly due to the type and number of cancers that exist and the fact that some have ineffective or no treatments available. Added to this, individual patients may respond differently to the same treatment, and patients who previously responded well may go on to develop resistance to the treatment, rendering it ineffective.

Our work aims to use animals, in combination with experiments that take place in the lab, to support the development of new treatments for cancer and address the unmet clinical needs of some cancers. The use of animals is an essential part in the process of developing new treatments for cancer. Animal studies enable us to replicate complex aspects of the disease in the entire biological system, including the interaction of cancer cells with other cells and organs in the body. They enable us to test the effect of new treatments on the disease (for example tumour growth) in a relevant and intact biological system. This work will enable key decisions to be made regarding which treatments should continue to be developed, and ultimately, which will make it into clinical trials, where they are tested in humans.
Our approach to the work ensures that this is done in the most efficient way possible, and that the benefit gained from every animal is maximised.

What outputs do you think you will see at the end of this project?

Work carried out under this licence may involve the development of new animal tumour models (creating incidences of cancer in animals, similar to the human condition) and potentially the improvement of existing animal tumour models. Collectively these will allow us to test the ability of new drugs to treat specific cancers in a manner that more closely replicates the human condition. As part of this project we hope to develop or improve 3-6 animal tumour models (for example using different types of cancer cell) and to test the effects of at least 5-10 new anti-cancer drugs using these models, or currently available models.

This project may provide important information to progress new cancer treatments through the phases of drug development. New test agents will be evaluated for their ability to treat cancer in animal models that replicate the conditions in human cancer. The information gathered will enable us to identify the most appropriate treatments to take forward to human clinical trials and, importantly, quickly determine which drugs should not be progressed any further.

In addition, this work will increase our knowledge of how new drugs work and will help us

to identify changes in the body that occur in response to the drug. We can use this further down the line to monitor responses in humans during clinical trials.

Data from studies under this project may be used to support patent applications and applications by clients for additional funding. Data produced may also support the design of regulatory studies for clients.

Who or what will benefit from these outputs, and how?

This project will generate important data in the development of new drugs. To assist with this, we will utilise our test agent dosing project licence to allow us to understand how drugs distribute in the body after dosing, how quickly they are eliminated, and the dosages that are well tolerated in rodents. This will aid in the design of dosing strategies for studies under this licence. Ultimately, work carried out under this licence is expected to result in the progression of new cancer treatments through various stages of drug development and ultimately into the clinic to treat patients with various forms of cancer.

Our focus on a science-led approach will enable key decisions to be made at each development stage on whether a test agent is likely to become a successful drug. This allows unsuitable drugs to be abandoned at an early stage and enables us to use the fewest number of animals possible per drug development programme. The identification of test agents as unsuitable for use in humans at an early stage of development will ensure a better success rate in the drug discovery process than has been seen previously in the pharmaceutical industry. Ultimately, this project will contribute to the successful development of new anti-cancer drugs, which may benefit patients with various forms of cancer who may otherwise have died from their condition.

The development of new or improved cancer models will enable us to test new drugs in as close an environment to the human condition as possible. This will increase the likelihood of patients benefiting from these new drugs.

How will you look to maximise the outputs of this work?

All studies are designed such that the outputs from each animal are maximised. Expert knowledge is gathered not only from within the preclinical (animal) team performing the animal studies, but from other teams within our company, or our clients' companies. This ensures that all relevant work that has been performed in the laboratory is taken into consideration when designing animal studies. The in vitro (in the test tube) team at our company are experts at analysing tissue and blood samples collected from animals, and they help with details of sample collection and storage to ensure that the samples are collected and stored in the best way possible. They are also experts at working with small quantities of samples, particularly small volumes of blood samples, meaning that they can often analyse lots of different biomarkers (a measurable indicator of a disease state or other physiological state) and test agent levels from each animal.

In addition, we will seek expertise from our established networks, to ensure that we make use of any new knowledge or incorporate better methods of performing animal studies. We will also use these networks to provide information and training to others on the models and techniques we use in our research. We will maintain good communication with managers of the animal facilities to ensure that any tissues from animals being killed that are not required for our work can be made available to other researchers if suitable.

Although there are times where we will not be able to share animal model information (for

example, where it would put us at a competitive disadvantage), we aim to publish or share our findings (especially control data) wherever possible.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most common type of animal used for developing new cancer treatments. Mice are well characterised, meaning that a lot is already known about how the body works in mice, and a large selection of different tumour cell types are already available for use in mice. This means that for the most common types of tumours, such as, lung, skin, breast and bowel cancer, cells are already available allowing us to easily evaluate new drugs designed to treat these kinds of tumours.

Adult mice will be used for the work outlined in this project licence as we want the biology of the animals to be fully developed as this will better represent the patients we aim to treat.

Typically, what will be done to an animal used in your project?

The majority of animals will be part of studies that aim to test whether new drugs can alter the growth rate of tumours. Typically, tumour cells will be implanted by injection under the skin on the flank of the animal, or, less commonly, cells will be injected into the mammary fat pad (breast) tissue, or directly into a vein. Except when injected into a vein, tumour cells will grow into tumours at the site of injection.

When injected into a vein, tumours cells will travel via the blood and form tumours around the body, most commonly in the lungs. The size of the tumours will be monitored as part of the experiment.

Tumours that grow just under the skin (flank and breast tumours) can be measured using callipers or simple hand-held imaging devices. Tumours will be measured a minimum of twice weekly, and the frequency of measurements will be increased during the faster growth phase. Tumours growing internally require monitoring by non-invasive imaging and by following the overall condition of the animal closely. For non-invasive imaging, animals will be anaesthetised as it is important that they remain still to produce the image. Animals will be imaged at least weekly when tumours are present, with frequency increasing during the faster growth phase. Tumours will typically be allowed to develop over a 4-12 week period, however, actual study duration will be dependent upon the type of cancer cell injected and how quickly they grow. Tumours are not expected to cause pain, however, any animal showing signs of pain will either be treated with a suitable dose of pain killer if appropriate for the scientific outcome of the study, or humanely killed.

As part of a study, animals will typically be dosed with anti-cancer agents (e.g. chemotherapies) over a period of several weeks or months and dosing will be initiated

typically when tumours reach a volume of 100 mm^3. Dosing will often take place on a daily basis, but this may vary depending upon the test agent. Drugs will be administered more commonly by the intraperitoneal (inside the body cavity), subcutaneous (under the skin) and oral (by mouth) routes and less frequently by the intravenous or intranasal routes. For intraperitoneal, subcutaneous and oral administration, mice will be held securely by a trained researcher for the dose to be administered. For intravenous dosing, mice will be placed briefly in a specially designed rodent restrainer, the tail will be warmed using warm water or a heat lamp to dilate the blood vessels and make them easier to see before administering the dose. For intranasal dosing, mice will be briefly anaesthetised and placed on their backs to allow the dose to be administered directly into the nostrils.

Less commonly, as part of some studies, animals bearing tumours may be given radiation treatment directed at the tumour either alone or alongside other anti-cancer treatments. Animals will be anaesthetised throughout the treatment and are expected to make an unremarkable recovery.

Blood samples, or small samples of tumour tissue, may be collected during some studies to measure levels of the test agent or to assess how well the drug is working. Blood samples are usually small in volume and are taken from a superficial vein such as the tail vein. Larger volumes of blood may be collected at the end of a study when an animal is under deep terminal anaesthesia. Small samples of tumour may be collected from live animals under anaesthesia using a very fine needle. Other non- regulated procedures such as passive urine collection (allowing the animal to pass urine naturally on to a clean surface so that it may be collected) may be performed.

At the experimental end point, animals will be humanely killed and tumours will be dissected and typically examined to look for evidence that the test treatments have worked.

What are the expected impacts and/or adverse effects for the animals during your project?

The most common adverse effects in the studies described in this project licence will arise from the tumours themselves or from the test agents. We are developing a scoring system that will enable us to monitor and ultimately minimise the level of suffering an animal experiences on our experimental protocols. Our scoring system will take into account the animals body weight, activity level, general body condition, posture and the condition of any visible tumours and will be used to classify the degree of suffering an animal experiences. We also take into account the duration of adverse effects and the cumulative experience of the animal. Therefore, an animal that experiences, for example, several short periods of reduced activity following dosing may experience mild suffering, but an animal that experiences several longer periods of significantly reduced activity that are accompanied by another clinical sign such as weight loss, may be experiencing more moderate suffering.

Metastases (cancer cells that have spread to other parts of the body) resulting from mammary fat pad tumours, and as a result of systemically (intravenously) administered cancer cells, can be difficult to detect and may cause the animals adverse effects as their size and number increases. Signs that an animal may have a number of internal tumours that are starting to affect normal functioning include reduced activity, weight loss (greater than 15% from starting body weight), poor body condition or poor posture. Again, we will use our in-house developed scoring condition to minimise the level of suffering an animal

may experience. Animals will be killed before they exceed moderate suffering.

Any animals with tumours large enough to impede normal mobility or their ability to eat and drink will be humanely killed.

The treatment of animals with known anti-cancer treatments or test agents designed to treat cancer may cause adverse effects, similar to humans. Following some treatments, mice may display reduced activity, hunched posture and fur bristling. For most treatments these effects should be short lived and mild, however some animals may experience moderate effects, which may include sustained weight loss of up to 15% of starting body weight and/or longer lasting reduced activity.

A small incision in mammary fat pad tissue may be required in order to visualise the tissue fully to enable us to give an accurate injection of tumour cells into the tissue. Incisions will be performed as a surgical procedure under recovery anaesthesia, and animals are therefore likely to experience some pain as a result of the incision. Pain relief will be provided 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 animal type)?

Mice:

Mild 30%

Moderate 70%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Wherever possible we use cell-based experiments, however, even where cells are used it is still necessary to use animals to understand the complex nature of cancer. Animals enable us to study the whole body, allowing us to study both how cancer cells interact with other cells and organs within the body and how cancer cells spread throughout the body. It is not possible to fully study this in isolated cells and/or organs.

Which non-animal alternatives did you consider for use in this project?

Our company regularly uses a range of in vitro (taking place in a test tube in the laboratory) methods utilising cells (typically cancer cells) to understand how a novel test agent might affect the cellular functioning of those cells. From these experiments we can prioritise test agents and only take forward those that have the desired effect, and therefore

those that look the most promising for the treatment of particular types of cancer.

Why were they not suitable?

Cell-based methods are useful to test the impact that novel test agents have on a cellular level, for one particular type of cell, but they do not model how cancer cells interact with other types of cells and organs, or how they spread throughout the body. In addition, cell-based methods do not test the effects that the body might have on a test agent, for example, how it is absorbed, distributed and excreted from the body, all of which can alter how effective the test agent might be. Testing a new drug in an intact biological system, such as a well understood rodent system, enables us to study the complex interactions of cancer with other cells/organs in the body, as well as monitoring how the test agent performs in an intact biological system. None of the alternatives mentioned can replicate this, although cell-based testing enables us to triage drugs and only take the best candidates forward for further 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 number of animals we will use throughout the five year lifespan of this licence has been estimated based on the number of cancer projects we have coming up in the next 12 months, combined with a prediction of likely demand of future projects for new clients over the remaining years of the licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have extensive experience in the design of experiments of the types in this project, which has given us confidence in the number of animals required to ensure that no animals are used unnecessarily, but also that the data generated is robust and reliable. Through experience, whilst we do tend to use a standard group size for the majority of our tumour studies (6 mice per group), we regularly refer to the PREPARE guidelines (https://norecopa.no/PREPARE) and make use of the NC3Rs Experimental Design Assistant (https://nc3rs.org.uk/3rs-advice-project-licence-applicants-reduction) to ensure that we are using the correct number of animals for every study. When designing experiments to look at the effect of novel test agents on tumour growth, where the test agent has not previously been dosed before, we look at published literature or client data to determine the variability observed with similar test agents and/or similar tumour models. We can then use the NC3Rs Experimental Design Assistant, or our own in-house developed tool to help determine the most appropriate group size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

An initial experiment using reduced numbers of animals is often conducted first of all,

which enables adjustments to be made before conducting the full experiment. Such experiments are referred to as pilot studies.

Data from pilot studies and previous experience are used to ensure that the numbers used are as low as possible, without compromising the robustness and reliability of the data. Within our company, a member of the wider team has generated a tool for performing power calculations and can be consulted as necessary to assist with study design.

Wherever possible, our in vivo scientists will be blinded to the treatment status of an animal, thus reducing bias. This enables more reliable information to be gathered from a smaller number of animals. Those who carry out analysis on samples (e.g. blood or tissues) collected during the study are also blind to the treatment status of the animal.

Baseline data (e.g. tumour size (where relevant), bodyweight) are recorded and animals are randomly assigned to treatment groups so there is no difference between the groups at the start of the study.

Good planning ensures that within any series of studies we can control for variability that might be introduced by external factors. To limit this variability we use animals of a similar age/weight range, test batches of test agent in the lab first, use the same source of animals and reagents, keep records of observations made and standardise as many components of an in vivo study as is practicable.

We will coordinate with other groups to share tissue including post-mortem tissues to reduce overall mouse numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
Animals will be housed in a purpose-built, state of the art facility, that is free of disease-causing organisms such as bacteria, viruses and parasites. They will have access to food, water and items that enhance their environment, such as tunnels, chew sticks and mezzanine levels to climb on. Our company staff and the animal care staff are competent in rodent welfare and will ensure that animal suffering is minimised. We aim to house mice in groups to promote normal behaviour. However, aggressive behaviour can occasionally result in animals being singly housed to prevent injury.

The most common type of tumour model we expect to use is the subcutaneous tumour model, which involves injecting cancer cells just under the skin (in the subcutaneous layer) in the flank of the mouse. This results in tumour growth at the site of injection. This is the simplest method available for inducing tumours in mice and results in the least harm to the animal as the tumour is self-contained within the subcutaneous layer and therefore cannot interact with organs or spread to other regions within the body. This technique can be used for different types of cancer; allowing the search for effective treatments for different

cancer types without needing to induce the cancer in the usually affected organ or tissue.

Other methods, such as mammary fat pad tumours and metastatic tumours will be employed when we need to answer more complex questions such as how cancer cells interact with different organs, how cancer spreads throughout the body, or how tumours respond to novel test agents in these different settings.

Why can't you use animals that are less sentient?

Adult mice are the lowest species of mammal that allow us to adequately study the complexities of human cancer. Due to the length of most studies, which span weeks to months to allow for tumours to grow, terminal anaesthesia is not a possibility. It is also important that we are able to monitor the behaviour of the animals in a conscious state as this allows us to monitor for adverse reactions to any test agents administered.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have a demonstrable history of refining our procedures to minimise harm to our experimental animals. This includes our basic practice of always using the smallest needle possible for dosing to minimise any pain and distress to the animal. We also investigate the suitability of test agents to be delivered via a route that causes the least harm. For example, we recently implemented this for a test agent, allowing us to switch from twice daily oral dosing (where the dose is administered via insertion of a tube down the throat) to dosing via the drinking water. The palatability of drug in drinking water will be carefully considered, and if appropriate, we may consider flavouring the water to increase its palatability.

General welfare monitoring is particularly important when conducting mice tumour studies. Animals undergoing tumour studies will be carefully monitored using our in-house body condition scoring system. Using this system, mice will be assigned scores based on body weight changes, changes in general condition (e.g. coat, posture and body condition), activity levels, condition of any visible tumours (colour and whether tissue necrosis or ulceration is present), as well as the number and size of any tumours (by non-invasive imaging such as the Peira TM900 handheld imaging device for measuring subcutaneous tumours or calliper measurements). Significant ulceration is not expected in the majority of animals as pilot work will be conducted to establish the rate of tumour growth that is least likely to result in ulceration at the tumour site. However, if ulceration develops, animals will be closely monitored and scored using our in-house developed scoring sheet, which will score ulcers as either dry or wet (exuding pus or clear fluids). Animals with wet ulcerated tumours persisting beyond 48 hours with no signs of improvement will be humanely killed. For dry ulcers, advice may be sought from the vet and minor interventions will be implemented if appropriate to allow the animals to continue the study.
Mice with increasing scores will be monitored more frequently to ensure that no animal suffers more than is necessary during each study. In our experience, assessing the whole body condition of the animal gives us much more information on the degree of suffering an animal experiences compared to measuring tumour diameter alone. Animals can often tolerate large tumours (particularly subcutaneous tumours) without a negative impact on overall wellbeing, making measurement of tumour diameter alone unsuitable for understanding the true impact on the animal, or when to kill an animal. Therefore, while our scoring system does take into account the size of tumour(s), this is considered collectively with the above factors to assess the impact of the tumour on the animal as a whole, meaning that we gain a more accurate picture of the animal's health and can

intervene early if welfare is significantly impacted. This in turn prevents the loss of valuable data and promotes maximum benefit from each animal that might otherwise have been lost when using a one-factor humane end-point.

Mice will be group housed where possible and provided with an enriched environment in order to minimise stress. Should pain relief be required, we will consider adding the pain relief to a palatable substance such as Nutella to allow the animals to take it voluntarily. During pilot work to establish mammary fat pad tumours in mice, we propose to inject cancer cells into two mammary fat pads of the same animal. This will allow us to compare variables such as the delivery vehicle (Matrigel) used to deliver cancer cells and the number of cancer cells injected to determine how they affect tumour growth in the same animal. This will allow us to reduce the number of animals used, particularly in the early stages of studies where we aim to determine the best conditions for each new cancer cell line.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use the Workman et al guidelines (Workman, P et al. Guidelines for the welfare and use of animals in cancer research. 2010. British Journal of Cancer. 102 p1555) to inform our experiments. In addition, we follow the PREPARE guidelines for the planning of studies (Smith et al., PREPARE: guidelines for planning animal research and testing. 2020. Laboratory Animals). LASA (Laboratory Animal Science Association) also has a range of published guidance documents with principles that can be applied to our animal studies which are found at https://www.lasa.co.uk/current_publications/.

In addition we will also refer to the following when conducting our work:

Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. (E Lilley and M. Berdoy eds.). http://www.lasa.co.uk/publications/

Smith D, Anderson D, Degryse A, Bol C, Criado A, Ferrara A, Franco NH, Gyertyan I, Orellana JM, Ostergaard G, Varga O, Voipio H (2018) Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Animal 51(1S): 5-57. doi: 10.1177/0023677217744587

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

When designing animal studies we consider the appropriate guidelines, including the guidance from LASA, the NC3Rs, and the PREPARE guidelines. This guidance will influence our study design.

173. Evaluating new targets for treating cardiovascular disease and cardiotoxicity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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 disease, cardiotoxicity, calcium handling, oxidative stress, therapeutic targets

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify molecules or proteins within the heart that are directly associated with cardiac contractile dysfunction either as a result of underlying chronic hypertension or resulting from the toxic effects of drugs or biomaterials. To target the identified molecules or proteins with a view to reducing or reversing the cardiovascular damage.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality globally and there is a strong need for improved and more targeted treatments. The left ventricle of the heart is responsible for pumping blood around the body and CVD can result in left ventricular dysfunction (impaired contractile function) from a number of underlying causes including high blood pressure and blood vessel damage. What is now also recognised is the contribution that certain drugs or devices can make to the overall CVD burden that leads to left ventricular dysfunction. Certain FDA-approved drugs and devices can have off-target effects that can lead to a condition termed cardiotoxicity (CTX). This is an umbrella term that covers a variety of toxic or damaging effects on the heart, many of the effects presenting in a similar way to those seen in CVD. Anti-cancer drugs in particular can have both short- and long-term toxic off target effects on the heart and this can ultimately result in heart failure. The underlying reasons for this off-target CTX are not well understood. As well as drug-induced CTX, patients with implants or devices have also been reported to present with varying degrees of off-target CTX as a result of the implant. In particular, patients with metal-on-metal (MoM) hip implants can suffer toxic off-target cardiovascular effects and this is due to cobalt ions being released over time from the MoM bearings and accumulating in the heart. Again, the mechanisms underlying cobalt-induced cardiotoxicity are not well understood. The progression of CVD or CTX is complex and variable but we know that three key pathways are likely to contribute (i) alterations in cardiac calcium handling (will affect heart contractility) (ii) increased inflammation (can contribute to heart tissue remodelling and dysfunction) and (iii) increased oxidative stress (can alter the energy available to the heart and lead to dysfunction). These may all contribute to the left ventricle of the heart becoming dysfunctional, with impaired cell-cell communication and an inability to pump blood effectively around the body. Access to human tissue to study these conditions is very limited. Therefore, this project will use a pharmacological rodent model of chronic high blood pressure to study novel potential therapeutic targets for the treatment of CVD and will use anti-cancer drug or cobalt treatments in rodents to study potential novel therapeutic interventions in drug- and device-induced CTX.

What outputs do you think you will see at the end of this project?

New potential targets for treatment of hypertension-induced left ventricular dysfunction will be identified.

Novel cellular mechanisms responsible for (i) anti-cancer drug-induced and (ii) metal-on-metal hip implant induced cardiotoxicity will be identified.

Research will be presented at national and international scientific conferences.

Research will be published in peer-reviewed scientific journals (at least one article per year) and, where appropriate, in newspapers for dissemination to the general public.

Who or what will benefit from these outputs, and how?

In the short-term, research information generated from our pharmacological models will benefit a number of research groups across the University of Strathclyde, Glasgow Caledonian University, University of Glasgow and Queen Elizabeth University Hospital. These are groups with whom we are currently collaborating and this will increase the capacity for research outputs, ensuring best use of the animal model. The cardiovascular, cancer and orthopaedic research community (Masters students, PhD students, research scientists, academics and clinicians) will benefit from our research outputs presented at national and international scientific meetings as well as networking events.

In the medium term, the cardiology, oncology and orthopaedic clinical community will benefit from research publications and ongoing collaborative projects. This work will inform upon potential treatments in clinical practice going forward.

In the longer term, people with CVD, cancer or MoM hip implants will benefit from the research findings should the novel targets identified from this project be taken forward to clinical trials.

How will you look to maximise the outputs of this work?

We will present our research at national and international scientific conferences and, follow up with publication in peer reviewed journals. Where appropriate, we will disseminate our research in news articles to the general public.

We currently have ongoing collaborations with cardiovascular and orthopaedic consultants and from the work in this project, will aim to develop and extend these collaborations to also include consultants in the field of oncology.

There are few network groups in the field of cardiotoxicity and we will plan to develop networking research groups (including basic scientists and clinicians) across Europe and further afield to share our knowledge from this work and build strong collaborations in this area

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rats or adult mice will be used for these studies. These are both well recognised models for assessing CVD characteristics as well as assessing the effects of drugs on the heart. The use of adults is essential to mimic the effects we would anticipate in adult humans.

Typically, what will be done to an animal used in your project?

To induce chronic high blood pressure, animals will be implanted with a minipump (a small oblong device) filled with a drug called angiotensin II. Age/weight matched controls will be implanted with minipumps filled with vehicle (we envisage using a very limited number of controls since the manufacturer has already established that vehicle-filled pumps have no adverse systemic effects). The pump will normally be inserted under the skin on the animal's back, parallel to the spine. This enables a constant rate of drug delivery and avoids multiple injections of drug. The pump will typically be left in place for four weeks to induce increased blood pressure. Typically, after four weeks the animals will have their blood pressure monitored using a tail cuff measurement and will then be put under

anaesthesia to have their heart function monitored by echocardiography (this is a non-recovery procedure).

To investigate the cardiotoxic effects of anti-cancer drugs, the same process will be followed, except minipumps will be filled with the appropriate anti-cancer drug of choice.

To investigate the effects of cobalt, animals will receive daily doses of cobalt chloride over a period of up to four weeks. Age/weight matched controls will receive daily doses of vehicle (e.g., sterile water). At the four week time point (if not before), blood pressure and heart function will be monitored as described above.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals receiving angiotensin II will gain less weight than an age-matched control and possibly lose some weight compared to their pre-treatment weight. They will develop high blood pressure and the contractility of the heart will be impaired. There can be some variation in the extent of these signs, similar to what would be observed in humans. If significant signs of ill health (due to chronic elevation of blood pressure) are seen, the animal will be used immediately with a terminal procedure (echocardiography) if possible or humanely killed.

Animals receiving anti-cancer drugs may gain less weight than age-matched controls and possibly lose some weight compared to their pre-treatment weight. There may be an increase in blood pressure and an impairment of heart contractility, however this is only likely to happen towards the end of treatment duration. Animals receiving cobalt chloride may experience some discomfort through repeated daily injections but this will be minimised through good handling practise and injection technique. The cobalt treatment at the dose and duration given is not likely to cause any obvious impact on animal weight gain. There may be a small impact on heart contractility but this is not likely to be significant and if it does occur, is only likely to be towards the end of the treatment duration.

Both blood pressure measurements and echocardiography are non-invasive procedures. Animals will need to be anaesthetised for echocardiography in order to keep them still to gain accurate monitoring of the heart. There may be temporary minimal discomfort during the induction of and recovery from anaesthesia but it is very short-lived.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect the severity will be moderate for the animals injected with angiotensin II, anti-cancer drugs or cobalt. Severity should be mild for control animals injected with vehicle saline solution.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to obtain cardiac tissue or cells from patients with cardiovascular dysfunction or heart failure since these patients are already very ill. Even if biopsies were possible, the amount of tissue available would be far too small to enable sufficient data to be generated. The use of an animal model ensures that the physiological features of cardiovascular disease in humans can be mimicked and also ensures sufficient heart tissue can be analysed. We can therefore study both the progression of heart disease and importantly, the effects of targeted intervention strategies.

Which non-animal alternatives did you consider for use in this project?

Where possible we will use commercially available cardiac cells (e.g. cardiac fibroblasts) to gain information however, the data we can retrieve from these is limited. We cannot source adult cardiac myocytes since these are not commercially available and we would be restricted to using neonatal or progenitor cells. We will use these alternatives wherever possible. At present, after checking databases such as Altweb and AltBIB we have not found non-sentient alternatives to our experimental needs.

Why were they not suitable?

For this project, it is essential to gain in vivo information on cardiac dysfunction for correlation to the human patient and we cannot gain that from isolated cells. Also any data examining progression of CVD or CTX in terms of mechanism of action must be performed on adult cells and tissue. Using the cardiac myocyte cell types that are commercially available restricts us to non-adult models and therefore poses major limitations in data interpretation. By using cells in general, we are also restricted in examining disease progression in any detail. We can only monitor relatively short-term effects. Cells kept in culture will change appearance and function over time and therefore any interpretation of data is more limited. Using cells in culture also means we cannot gain information on whole heart function which is far more complex.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated our animal numbers based on our previous work using a surgical model of heart disease, as well as our previous work using infusion of cardiotoxic anti-cancer drugs or cobalt. We have used the minimum number of animals we need to show meaningful statistical differences between experimental and control groups.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

NC3Rs EDA as well as advice from our University statistician, basing the numbers on our previous experimental work. We have ensured that as much cardiac material as possible is used from each animal to enable several experimental outputs and a reduction in the overall numbers needed. The heart tissue from each animal will be sectioned into different parts, each of which can be used for different types of assessment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For some experiments, tissue will be divided into a number of samples and prepared in specific solutions for long term storage. This will allow experimental analysis of a number of different parameters over a period of time from one animal. It will also enable several investigators to work with the same animal. If we are isolating cardiac myocytes from an animal, there will be a sufficient number of cells to ensure these are given to several investigators on the day of isolation and this will ensure multiple outputs from one animal. We will also ensure that, where appropriate, we share material from each animal. For example, researchers working on pulmonary tissue and vessels can dissect these from our rats and researchers working on brain can dissect this from either mice or rats once we have the heart removed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a rat or mouse model of chronic Angiotensin II infusion. Angiotensin II is a hormone produced in the body that is important in regulating blood pressure. The infusion model in rats or mice is a well recognised model and will mimic the progression of increased blood pressure and resulting effects on the heart that are seen in people with cardiovascular disease. Our previous work has used different surgical animal models of heart disease however, these have been invasive and there is always a risk of animal mortality due to the surgical procedure. The Angiotensin II model involves a straightforward placement of a mini-pump under the skin of the animal and this circumvents the need for invasive procedures with less likelihood of adverse effects due to surgery.

For studying anti-cancer drug-induced cardiotoxicity, we will also use a mini-pump implant and, based on previously published work by ourselves and others, will use the lowest concentration of anti-cancer drugs known to induce an effect on the heart. For cobalt-induced effects we will typically use daily injections of cobalt chloride, again at the lowest concentration known to induce an effect on the heart. This will ensure there is the lowest level of harm and distress to the animals.

Why can’t you use animals that are less sentient?

For this work we need to mimic the effects that are seen in human beings with heart disease or human beings who have had chronic treatment with anti-cancer agents or exposure to cobalt. These are invariably adults, usually older adults. A model that is less sentient would not be relevant to this study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use refined procedures that have been developed by us and by colleagues at other institutions and we will monitor the animals daily. Animals are given pain relief for mini-pump implant and are monitored daily post-operatively until fully recovered. Animals will normally be housed with a 'buddy' or "buddies" (conspecifics) for the duration of the experiment to improve overall welfare. If any of the experimental animals show signs of ill-health and deterioration that cannot be reversed, they will be used in early terminal procedures (echocardiography and tissue harvesting) or will be killed humanely.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Any relevant NC3R publications within the cardiovascular field will be adhered to. We will also take regular advice from colleagues who are working with the same models at other institutions in the UK.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will check the NC3Rs website regularly and will gain information from our Named Information Officer (NIO). We will attend any relevant NC3R research days or conferences.

174. Prenatal Therapy for Fetal and Obstetric 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

therapy, fetal, genetic disease, stem cells, gene therapy

Animal types	Life stages
Sheep	pregnant, neonate, juvenile, adult
Guinea pigs	pregnant, embryo, neonate, juvenile
Mice	embryo, pregnant, neonate, juvenile, adult
Rats	embryo, pregnant, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We are developing safe and effective methods of delivering treatments using stem cells, genes and drugs/proteins to the fetus and placenta, to reduce disability and death from congenital diseases such as thalassaemia, haemophilia, metabolic liver conditions and obstetric conditions such as fetal growth restriction, pre-eclampsia, gestational diabetes, preterm premature rupture of the membranes and preterm birth.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many fetal and placental problems are related to genes. Genetic abnormalities may be parentally inherited e.g. mutations causing thalassaemia, a disease of the blood causing anaemia and fatigue in affected people. Alterations in placental genes and/or blood supply can produce abnormal protein levels, which may severely limit fetal growth. Genes can be packaged into viruses or artificial carrying systems (vectors), which insert into host cells. The incorporated genes work within the cell to correct abnormal gene function or produce a therapeutic effect (gene therapy). Stem cells can regenerate, providing never-ending supplies of specialist cells, and can be modified by gene therapy before transplantation (stem cell gene therapy). Alternatively, proteins or drugs can be provided to the mother or fetus to produce a therapeutic effect. Delivering proteins, stem cells or gene therapy early in pregnancy, before the immune system has fully matured, may prevent the baby from developing antibodies against the vector, allowing it to function for longer.

We are targeting a range of life-threatening conditions that begin before birth, for prenatal treatment. Thalassaemia is the most common genetic disorder in many countries, and many affected individuals die in the womb or as teenagers. Metabolic liver disorders are associated with significant morbidity and mortality in the neonatal period (e.g. Ornithine Trans-Carbamylase Deficiency) or later in life (eg Wilson's disease). Approximately 1:10 babies fail to achieve their growth potential in the uterus (growth restricted) usually because of a lack of maternal blood flow to the uterus and placenta, limiting the supply of nutrients and oxygen. Sometimes mothers develop gestational diabetes leading babies to put on too much weight, a risk associated with stillbirth and preterm birth. Alternatively in some pregnancies, the membranes around the baby rupture early leading to problems of lung development and preterm birth. In these pregnancy conditions, fetal growth restriction, gestational diabetes and preterm premature rupture of the membranes, babies may be born preterm leading to conditions such as chronic lung disease, infections or cerebral palsy. Delivering treatment to the fetus or mother to treat or prevent these pregnancy conditions requires excellent diagnosis and safe, accurate imaging.

If successful, these therapies will transform fetal medicine and neonatal care, since there are currently no effective treatments for these conditions. Parents of affected fetuses are faced with two options: to terminate the pregnancy or to continue, knowing the baby will be severely affected growing up, or may die before birth. Prenatal therapy presents a third option to these parents.

Our aims are fourfold:

To identify the best stem cells, viral vectors and drugs, and determine when and how they should be given.

To deliver these therapies to animals with congenital or generated models of diseases such as thalassaemia, metabolic liver disease,, fetal growth restriction and gestational diabetes to establish which are the most effective for treatment.

To determine the long term effect of these therapies on fetuses born after therapy.

To develop new and improved ways to safely image the fetus in order to correctly diagnose and treat within the womb and to assess the effects of treatment.

Vectors, stem cells and proteins will be studied in the lab on cells, before being used in any animals, to reduce animal numbers. For these studies we will use congenital mouse models of human disease (up to 3950 animals in total) to demonstrate efficacy of therapy. We will also use guinea pigs (up to 300 animals) because their placental development is the most similar to humans when compared to other mammals, and rats (up to 400 animals) in which pregnancy complications can be generated. Finally, sheep (up to 250 animals), will be used as a way to demonstrate safety and efficacy as they have many similarities to humans in terms of fetal size, dependent on the breed, and development and a longer length of pregnancy than smaller animals such as rabbits. Where possible, vectors, stem cells and/or proteins will be delivered using clinically relevant minimally invasive ultrasound guided injection, that reduces animal suffering compared to large surgical cuts. We will use implanted catheters to monitor heart rate and blood pressure continuously, and which can transmit the data wirelessly to a nearby computer, allowing the animal to roam freely in their enclosure.

What outputs do you think you will see at the end of this project?

Many conditions that affect the fetus (baby in the womb) are currently untreatable. Collectively these conditions lead to a large burden of disease. Some of these conditions are genetic and life-threatening to the fetus and the newborn, such as haemophilia and thalassaemia that affect the blood, or inherited liver disease. Others severe conditions are obstetric disorders that develop in pregnancy. These include severe fetal growth restriction (FGR) where the baby is extremely small, gestational diabetes (GDM) where the baby grows too big, or preterm premature rupture of the membranes (PPROM) leading to preterm birth (PTB) before 37 weeks of gestation.

For many of these disorders, the only option for parents when a diagnosis is made, is to terminate the pregnancy or to accept an affected child. Our work will provide parents and healthcare givers with a third option, namely to treat the fetus before birth.

Preliminary work in small animal models has demonstrated cure of some severe genetic disorders using gene therapy before birth. Gene therapy involves the introduction of normal genes into cells in place of missing or defective ones in order to correct genetic disorders. Prenatal gene therapy would aim to provide treatment before birth. There may be risks involved in giving gene therapy to the whole fetus and this project licence will provide data on what those risks are. Alternative prenatal treatments include giving stem cells either on their own or in combination with gene therapy to the fetus. This project licence will therefore also tell us how effective these alternative treatments may be at curing severe genetic disease.

For obstetric disorders, work in small and large animals has shown that giving gene therapy to the mother's blood supply to the womb can improve fetal growth before birth. This project licence will provide us with safety and efficacy data to support a clinical trial of this new type of therapy. We will also find out how effective are patches to seal the fetal membranes, to prevent preterm birth after fetal treatment or PPROM, and if we can improve excess fetal growth in gestational diabetes.
Finally all diseases in the fetus need better ways to image and make a diagnosis. We will be testing out the accuracy and safety of new imaging techniques to improve our ability to identify diseases before birth.

Overall the work in this PPL will advance the field of knowledge further in fetal therapy, with significant outputs in terms of publications, patents, conference presentations and

data for regulators to support the clinical development of new devices and drugs for fetal treatment.

Who or what will benefit from these outputs, and how?

The likely beneficiaries of this research are patients, i.e., both parents and fetuses/newborns, that are subject to a pregnancy complicated by genetic, obstetric and vascular disorders like Fetal Growth Restriction (FGR), Gestational Diabetes (GDM), Haemophilia, Metabolic Liver Disease, preterm premature rupture of the membranes (PPROM) for example.

For FGR, we are currently developing a first-in-human clinical trial (phase I/IIa) to assess the safety and efficacy of a new drug to improve growth of very small fetuses before birth. FGR complicates around 8% of all pregnancies and is severe in around 1 in 500. We have already demonstrated in FGR sheep and guinea pig pregnancy that this drug improves growth without any safety concerns. The pharmaceutical regulatory authorities (EMEA and MHRA) have reviewed our plans to translate the VEGF gene therapy into man. Before we do so we have been advised by the Medical Research Council to whom we have successfully applied for funding, to test out the proposed clinical Drug Product in further FGR guinea pig pregnancies. This will generate further safety and efficacy data with the Drug Product that will be used in the clinical trial and to better understand the dose response.
Further data on the mechanism of action in mouse models of FGR will improve the chances of this therapy getting through the regulatory procedures. The clinical trial would commence in 2 years' time if successful. The beneficiaries would be fetuses, newborns with FGR and their parents.

For genetic diseases such as haemophilia and thalassaemia, we are working with collaborators who have small and large animal models of disease. If cure is demonstrated, we will apply for stem cell developmental pathway funding support (MRC) to develop the toxicology package and first in man study, with a timeline of 5 years. We will also investigate the therapeutic potential of stem cell and gene therapy in congenital metabolic liver disease including disorders of the urea cycle (e.g. Ornithine Trans-Carbamylase Deficiency; OTCD), the arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome, mucopolysaccharidosis (MPS) and Wilson’s disease. Treatment of these conditions is currently limited to newborn gene or stem cell therapy if available, or palliative enzyme replacement which does not improve outcome. Because the pathology of these conditions begins before birth, newborn treatment is too late. Increasingly clinicians are considering treating the fetus before birth. This is particularly because non-invasive prenatal diagnosis is becoming available for many congenital disorders, which increases the safety of diagnosis. Mouse models of OTCD, ARC syndrome and MPS are available locally, and we have established a collaboration with a US group that have extensive experience with fetal gene therapy in murine Wilson’s disease. If a beneficial effect of stem cell and gene therapy is demonstrated in this setting (timeline 3 years), we plan to investigate these interventions in large animal models of liver disease (sheep).
For amniotic membrane rupture, currently during fetal surgical procedures we use small needles or telescopes called fetoscopes to apply therapy to the fetus. It is possible to plug the defect in the amniotic membrane after surgery with human platelets. But this is associated with a 16% stillbirth rate and the seal often does not work, leading to ruptured membranes or PPROM. This complicates around 30% of fetal surgeries and results in preterm birth. We would like to develop a better approach using a novel protein system that rapidly coalesces with amniotic fluid to form a seal. If successful, we would need to

get regulatory approval for this approach and test out safety in humans, with a timeline of 5-8 years.

To improve fetal therapy we have developed some novel needle tracking devices which can more easily identify the needle tip when it is placed into the fetus during ultrasound-guided fetal surgery. We are also studying a new way to image small blood vessels in the placenta, to improve diagnosis and treatment of placental disorders in twin pregnancies.

How will you look to maximise the outputs of this work?

We aim to maximise the outputs from this work by collaborating with a wide variety of experts both in the UK and internationally to avoid duplication of effort. We will be seeking feedback/inputs from the regulatory authorities such as MHRA at all steps towards clinical translation with the ultimate aim of taking these therapies and devices into the clinic.

We will disseminate the knowledge gained from these studies in conference proceedings, publications and will also publish unsuccessful experiments/methodologies. Our research team frequently publishes in high impact journals (Stem Cells Development, Molecular Therapy etc) and we have regular meetings with a patient public engagement group via the GIFT-Surg project (Wellcome Trust/Engineering and Physical Sciences Research Council funded) at which we present the findings of the research into optimising imaging and prenatal therapy. With them we take into consideration their advice on how best to translate findings into the clinic.

Species and numbers of animals expected to be used

- Sheep: 250
- Mice: 3950
- Rats: 400
- Guinea pigs: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using various types of animals (mice, rats, guinea pigs and sheep) to develop novel clinical therapies to treat serious congenital (inherited) disease and diseases affecting pregnancy. We have chosen specific types of animals because they provide particular advantages and have similarities to human pregnancy and fetal development in the womb before birth.
Many genetic (inherited) models of human disease can be generated in mice making it possible to test out how effective a novel treatment might be, before moving to clinical trials in humans.

Specific types of pregnancy complications such as fetal growth restriction can be generated in mice, rats and guinea pigs. In addition, the guinea pig placenta has the closest structure to the human placenta of all small mammals. This means that experiments in guinea pig pregnancy provide invaluable data on how effective a treatment

might be on the human placenta. Such data can also be used to inform the regulatory authorities about the safety of a new treatment.

Finally pregnant sheep have a much longer length of pregnancy than rodents (maximum 65 days) which is closer to the length of pregnancy in humans (145 days in sheep versus 266 days in humans). They also tend to have only one or two offspring, similar to humans, whereas rodents usually have at least 3-5 pups. The physiology of fetal development in the sheep is also very similar to human fetal development. Therefore data on fetal development, fetal growth and gestational age at delivery are highly relevant in answering how safe and effective a potential clinical treatment might be in humans.

We plan to study pregnant animals as we are developing treatments for diseases of the fetus, placenta and mother in pregnancy. We shall also study the offspring of these treated pregnancies to get important data on long term safety and effectiveness.

Typically, what will be done to an animal used in your project?

Typically animals will undergo procedures under general anaesthetic. Before a procedure, sheep will not eat overnight to reduce the chance of vomiting. For guinea pigs where there is a risk of vomiting, access to food will be restricted for a few hours only to limit any harms. Animals will have free access to water. Pain relief will be given to aid recovery of the animal after surgery. This will include local anaesthetic injected subcutaneously to the wound and buried sutures to reduce pain. Animals will receive intravenous antibiotics to reduce the risk of infection.

Specifically in Protocols 1 and 2 we will study the effect of stem cells, gene therapy or drugs/proteins that can improve their effect in pregnant sheep (ewe) or fetal sheep. The stem cell and gene therapy treatments will be delivered to the ewe or fetus using either ultrasound image-guided needle injection or surgical procedures under general anaesthesia. In Protocols 1 and 2 some ewes with their fetuses will be humanely killed before birth.

Some ewes will deliver their lambs and these lambs will undergo further minor procedures in Protocol 3 to understand about the safety and effectiveness of the stem cell or gene therapy after birth (blood sampling, liver and bone marrow biopsy, immune response, semen collection). These procedures are either associated with minor short-term pain (blood sampling, immune response, semen collection) or are performed by needle biopsy under local anaesthesia (liver and bone marrow biopsy). All lambs will be humanely killed by two years of age.

For Protocol 4 we are studying the effect of novel treatments in fetal sheep to seal the amniotic membrane around the fetus in utero to try to prevent preterm birth. Under general anaesthesia we will create small holes in the amniotic membrane either using small telescope instruments or through surgery. We will then test sealing of the hole / defect using tissue-engineered cells in combination with drugs, materials, nano-particles or other substances to improve stem cell engraftment or patch incorporation into the membrane. In Protocol 4 ewes and their fetuses will be humanely killed before birth.
In Protocol 9 we will test out novel minimally invasive imaging, sensing, and tracking devices in pregnant sheep to improve the safety and effectiveness of fetal therapy. Procedures will be done under general anaesthesia with analgesia and antibiotics to prevent infection, and all ewes and their fetuses will be humanely killed before birth.

For all the sheep protocols (1-4 and 9), image-guided needle injections take only a few minutes to perform and rarely cause maternal or fetal post-operative pain, post-operative infection and stress. The surgical procedures will involve either placement of catheters through small holes or by making incisions in the animal to deliver the interventions. This is associated with maternal post-operative pain, infection and stress in <5% of animals. The fetal complications are infection, procedure related mortality of <5% and preterm birth of around 5-10%. Sheep will receive pain-relief and antibiotics during and after the procedures to reduce these harms.

Specifically in Protocol 5, we will generate obstetric complications such as fetal growth restriction (FGR), pre-eclampsia (PET) and gestational diabetes (GDM) in pregnant mice, rat or guinea pig dams, either because they occur through a genetic mutation in the mouse or by techniques such as reducing or increasing dietary intake, reducing oxygen level or reducing blood flow to the uterus, or administration of some drug substances. Under general anaesthesia we will then test out gene therapy or drugs that may increase fetal growth, reduce maternal blood pressure and poor sugar metabolism to try to improve both maternal and fetal outcome. In Protocol 5 some dams and their fetuses will be humanely killed before birth.

In Protocol 6, we will study pregnant mice dams with genetic mutations that recapitulate severe human genetic diseases. These genetically altered pregnant mice will be bred under Protocol 8. Under general anaesthesia we will then deliver stem cells to the fetuses to try to improve both maternal and fetal outcome. In Protocol 6 some dams and their fetuses will be humanely killed before birth.

In Protocols 5 and 6 minor degrees of maternal post-operative pain, post-operative infection and stress may occur in <5% of animals. The fetal complications are procedure related mortality, infection, vector or stem-cell related toxicity, pain and stress, which are likely to affect up to 10% of animals.

Some dams from Protocols 5 and 6 will deliver their pups to allow long term monitoring of the safety and effectiveness of treatments in these offspring under Protocol 7. Animals will undergo blood sampling or intravenous catheterisation which carries minimal harm and pain. Some animals may undergo ultrasound, magnetic resonance, X ray and bioluminescence imaging procedures under short general anaesthesia which is associated with minimal harms such as stress. Some animals may undergo short surgical procedures under general anaesthesia to place catheters for monitoring or one liver biopsy. Potential associated harms are post-operative pain, infection and stress in <5% of animals which will be relieved using analgesia and antibiotics. Pups will be followed up to one year of age before being humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Major maternal complications are short-term and are related to anaesthesia such as cardio-respiratory depression, inhalation of regurgitated rumen contents and dehydration. Minor degrees of maternal post-operative pain, post-operative infection and stress may occur medium term (eg 1-2 days). Long-term effects (weeks to months) are not expected to occur.

The short-term fetal complications are procedure related pain, mortality and miscarriage (most commonly due to infection). The fetus may experience medium or long-term adverse effects related to gene therapy or stem cell related toxicity, pain and stress.

The Named Veterinary Surgeon will be advised of any major adverse events and advice will be taken on suitable management.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities are moderate for most protocols in sheep, rats, mice and guinea pigs that undergo surgical interventions. For the protocols that include neonates born from other protocols, the severity is mainly mild.

On the basis of past experience, we anticipate that maternal complications in sheep, guinea pigs, rats and mice will occur in less than 5% of procedures. Fetal survival varies according to the protocol and will be >95% in large animal experiments, >85% for guinea pig and around 50-60% for transgenic mouse experiments.

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 consider the use of animals to be unavoidable. It is not possible to test our hypotheses in cell culture since we are testing an effect on for example, fetal growth and size, or for example, an increase in circulating concentrations of clotting factors, rather than individual cells in culture. Similarly data on safety of our potential therapeutics needs to be done in animals so as to measure both short term (eg bleeding) and long term (eg apoptosis, inflammation, organ damage) effects. This needs to be done in live animals to achieve our aims.

Similarly, the use of animals to establish device and technology performance of novel technologies is critical to inform development of next-generation medical technologies and to prove functionality prior to translation to the clinical arena. Our benchtop testing using tissue-mimicking phantoms is world- leading, yet elements of device function within the biological environment remain extremely challenging to satisfactorily assess without the use of animals. This includes the performance of elements in the blood pool, the system performance as well as assessing components in as close an environment to that of a clinical situation as possible. Only once such benchtop experiments are performed will we move to deliver our technologies to animal studies in vivo. Likewise, these animal studies are required before devices and technologies are delivered to human patients.

Which non-animal alternatives did you consider for use in this project?

We will be optimising drugs, vectors, stem cells and devices for this project first outside animals (in vitro culture, ex vivo tissues, tissue-mimicking phantoms) as much as possible, so as to reduce the use of animals.

All gene transfer vectors are extensively tested in vitro first, to determine if the vectors can infect cells and express the transgenic proteins. Initial testing is in immortalised mouse or human cell lines followed by testing in primary mouse cells and, where possible, primary human cells, after which there is testing in tissues taken from sheep or guinea pigs before moving to in vivo vectors application.
Similarly, with stem cells we will test these first in culture to determine their growth and differentiation characteristics. Only then will we move to deliver these to animals in vivo.

For the studies with devices we are able to perform detailed and comprehensive assessments of the performance of systems prior to approaching animal work. We will first test devices out in tissue- mimicking phantoms that have been 3D printed from organs, or use animal or human tissues. For example we have access to human placenta after birth via our hospital in which we have ethical approval to test out devices in the placenta ex vivo. We have also constructed phantoms using chicken or beef tissues from butchers.

Why were they not suitable?

Non-animal alternatives are not suitable for all our aims because our experimental readouts can only be obtained from living beings.

It is not possible to test our hypotheses in cell culture since we are testing an effect on for example, fetal growth and size, or for example, an increase in circulating clotting factors, rather than individual cells in culture. Cells in culture can give us focussed information on the effectiveness of a potential therapeutic in specific cells but not in the whole organ or animal.

For devices, we will need information on safety in live tissues such as tissue damage, inflammation, apoptosis thus ex vivo tissue is unsuitable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 animal used include pregnant adults and lambs or pups born from those pregnancies that undergo postnatal follow up.

Sample size calculations have been conducted with statistical help from our clinical trials unit for experiments on fetal growth restriction, and will be based on previous observations of uterine artery blood flow and fetal size in sheep and guinea pigs. The calculation will include consideration of litter number and litter sex and will be based on the number of dams used rather than the number of fetuses treated, as recommended. This is because in these experiments the unit of treatment is the mother/dam and not the fetus. Where

possible, larger experiments using a contemporaneous control group are preferred to smaller experiments where a control group is used from the past.

In large animal experiments where the unit of treatment is the fetus and not the dam/mother, an initial experiment using five dams per group will be performed if we do not have data from previous experimental analyses for these transgenic animals. A retrospective power calculation will then be performed to test whether sufficient animals have been used per group. The experiment may then be repeated in order to provide sufficient power. The statistical test used will take into consideration factors such as litter size, and the number of homozygote affected fetuses or neonates. Generally where we are using gene therapy containing a human protein, the animal does not express the human protein. Therefore we expect an "all or nothing" response where either all animals express or none do. In this case n=10 animals is usually sufficient.

For transgenic small animal experiments we have done calculations on effect sizes based on previous data in these animal models. We will compare with contemporaneous control animals that do not have the genetic abnormality but have the same background.

For imaging and device and technologies developments, an individual animal can provide a wealth of data. Thus by carefully constructing operating procedures and protocols we are able to minimise the numbers of animals used for each experimental series. An initial series of experiments aiming to show technology functionality and informing future device iterations will be performed for each major technology development. 4 animals per group will be used for this initial experiment. Device design and functionality will be informed by these and new iterations extensively tested on the benchtop before returning to animal work. Experimental protocols will be performed on up to a further 10 animals to establish functional reproducibility and measurement consistency in vivo, thus establishing robust proof-of-principle and enabling planning for translation to the clinic.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible, larger experiments using the same control group are preferred to smaller experiments where a control group is included each time.

In transgenic animal experiments, where we do not have pilot data, we will perform an initial pilot experiment using five dams per group and a retrospective power calculation will then be performed so as to refine /reduce the number of animals being used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have longstanding experience of working with pregnant animals for experimental use and will use our optimised breeding programs, that take care to ensure that animals are only bred when they reach their optimal weight and age.

For device and technologies developments, an individual animal experiment may be used to test a variety of devices in a carefully conducted series of experiments so as to minimise the numbers of animals used. Where possible we will also take tissues from a few animals (sharing of tissue) to use to test out devices ex vivo in the lab, so as to reduce animal numbers.

In addition to adopting efficient breeding strategies, we will maximise on the experimental readouts that we can obtain from each animal; such as harvesting/sampling tissues and organs widely and banking them so that they may be used for multiple studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We propose using the sheep fetus because the similarities between the sheep and human fetus in size and function make it the most widely used animal for studying fetal physiology and give clinical relevance to these experiments. Much developmental work has been done using cultured cells and rodents, but these data require validation before translation into clinical practice. However, issues such as gene therapy toxicity, length of gene expression and distribution of the resulting expressed protein can often not be addressed in small animals. We have extensive experience in working with pregnant sheep (22 years) and guinea pigs (14 years). During this time, we have refined the experiments to reduce morbidity, for example in sheep and guinea pigs we will minimise the animal suffering by using telemetric "remote" monitoring of implanted flow probes or catheters to measure blood flow or cardiovascular indices longitudinally. This allow animals the freedom to move around their environment without being troubled by monitoring devices. We have also developed ways to enhance the speed of recovery after general anaesthesia in sheep and guinea pigs by keeping our procedures short and by providing dietary enhancements (fresh lettuce for guinea pigs and nuts for sheep) to encourage eating after the anaesthesia.
For clinical application it is important to find minimally-invasive injection techniques to deliver gene, cell or drug therapy safely with minimal damage to the mother and fetus. This can only be achieved in a large animal of similar size to humans such as the sheep. Because these delivery techniques will be designed to produce the least trauma to the mother and fetus, there will be minimum suffering to the animals involved. It is likely that a total of 250 sheep will be used for this project over 5 years. In our experience fewer than 1 in 10 mothers or fetuses will experience some adverse event such as bleeding, infection or miscarriage. This has been achieved by paying attention to close clipping of the fleece and triple cleaning of the abdomen to reduce infection, providing intravenous fluids during surgery, and ensuring good analgesia through local subcutaneous anaesthetic to skin wounds. Lambs born after prenatal gene or stem cell therapy are usually healthy, and show no signs of having undergone any procedure. We will encourage the mother to suckle the lambs, but if unsuccessful we have access to frozen colostrum and can bottle feed them.

Fetal growth restriction (FGR) and gestational diabetes can be created in small animals and the underlying placental problem in these affected animals is similar to the human condition. It is not possible to study the effect on fetal growth in computer simulations or in cell culture and therefore animals are required. Suffering will be minimised by having a relatively mild nutrient restriction, in which we have found that animals often still have food

remaining in their cages. Although nutrient restricted guinea pigs are housed singly to ensure that the dietary intervention is correctly applied, we place the cages together so that the animals can see each other. We also provide straw to encourage nesting behaviours. The pups born after the FGR pregnancies are only moderately small and they catch up neonatally.

Pre-eclampsia mouse models experience moderate high blood pressure and some protein in the urine but no other adverse effects. Their pups may be slightly growth restricted but catch up postnatally. Gestational diabetes mouse models have pups that are slightly larger than normal pups, and they may develop moderate high blood pressure in adulthood, but no other adverse effects. We will try wherever possible to use less invasive techniques such as oral delivery rather than intravenous or intraperitoneal injection, and to avoid anaesthesia for non-invasive imaging such as echocardiography using ultrasound in guinea pigs. Refinements to our experiments in mice and rats include ensuring that general anaesthesia and surgeries are short, for example we will try to keep the total time for stem cell injections to no more than 30 minutes as we have found this reduces the fetal loss rate. We will also give local anaesthesia subcutaneously to abdominal wounds to improve pain relief and prevent the animals from pulling at the wound.

Heterozygote mice that carry the trait for congenital blood disorders do not show any abnormalities apart from a mild anaemia (thalassaemia mouse) and slightly prolonged clotting time (hFIX mouse). Homozygote thalassaemia mice (that carry both abnormal traits such as the humanized mouse model, are anaemic at birth but can be rescued by newborn blood transfusion, as in the human condition.
Homozygote haemophilia B mice are well unless they undergo trauma or surgery, in which case they require hFIX protein supplementation.

Heterozygote mice that carry the trait for metabolic liver disease (e.g. the ARC syndrome, MPS, Wilson’s disease) appear to be normal and exhibit no clinical (including ascites), biochemical or histological evidence of disease. Heterozygous females from the mouse model of OTCD are also normal (no clinical, biochemical or histological evidence of disease), but a few die soon after giving birth (due to the increased metabolic demands of the perinatal/postnatal period). Heterozygous OTCD mothers will be fed wet mash prior to parturition (from E16 onwards), and will be closely monitored in the early postnatal period (1-2 weeks). If there is any evidence of distress and/or weight loss (more than 10% of body weight) dams will be humanely killed.

In order to realistically demonstrate potential clinical functionalities, imaging and tracking technologies and devices must be tested in morphologies and dimensions similar to those found in the clinic. Although small animal models (e.g. rodents) may provide highly valuable data on limited aspects, larger mammalian models are required for complete validation, with distances and organ dimensions similar to that of humans. Sheep have a vascular and circulatory anatomy comparable to that of humans and thereby provide an excellent model. All our device work will be performed with the highest regard to animal welfare and every effort will be taken to minimise suffering and pain. Device experiments will be performed under terminal anaesthesia, with local anaesthesia used for skin incisions to minimise tissue reaction. The principles of the 3Rs (Replacement, Reduction and Refinement) will be adhered to. In particular, benchtop (non-animal and tissue-mimicking phantom) models will be used to the greatest extent possible before we move to animals, and the number of animals used will be kept to a minimum.

Why can’t you use animals that are less sentient?

As a majority of the experiments proposed need to be performed in adult pregnant animals to develop prenatal therapies, we are unable to use them at a less sentient stage of their life. Where possible, some experiments will be performed under terminal anaesthesia but for many we need to follow up animals after interventions to understand the effect of the intervention on their wellbeing, to study pathology and examine long-term outcomes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All experimental animals will be monitored 3 times daily on weekdays and 2 times on weekends. Pre- and post-operative analgesia (pain relief) regimens will be an integral component of all procedures, to keep animal suffering to a minimum. We will give antibiotics to reduce the risk of infection in animals that undergo major surgery. We will give local anaesthetic under skin wounds to reduce pain and suffering, and bury the stitches under the skin to avoid irritation.

We will try wherever possible to use less invasive techniques such as oral delivery rather than injecting the veins or peritoneal cavity. We will as far as possible avoid anaesthesia for non-invasive imaging such as ultrasound of the heart (echocardiography) in guinea pigs.

In each protocol, animals will be humanely killed if there are any signs of distress and/or significant reduction in body weight to avoid any distress secondary to deterioration in liver function or anaemia for example.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the published ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines) to ensure that experiments are conducted in the most refined way.

For example we will use published guidelines to develop the study design (Bate ST and Clark RA (2014). The design and statistical analysis of animal experiments. Cambridge University Press. https://www.cambridge.org/core/books/design-and-statistical-analysis-of-animal- experiments/BDD758F3C49CF5BEB160A9C54ED48706)

We will perform sample size calculations, randomly allocate animals to intervention or control groups, analyse data blind to intervention, use prior setting of outcome measures, use SAMPL guidelines to analyse and present data statistically (Basic statistical reporting for articles published in Biomedical Journals: The “Statistical Analyses and Methods in the Published Literature” or the SAMPL Guidelines; Michel MC, Murphy TJ and Motulsky HJ (2020). New author guidelines for displaying data and reporting data analysis and statistical methods in experimental biology. Mol. Pharmacol. doi: 10.1124/mol.119.118927).

We will report experiments according to ARRIVE version 2.0 (Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0 (Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0 https://journals.plos.org/plosbiology/article? id=10.1371/journal.pbio.3000411)

For best animal welfare we will follow the NC3Rs guidelines for all the rodent animals that we intend to use:

https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry-guinea-pig
https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry-mouse
https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry-rat
For sheep we will refer to RSPCA guidelines as NC3Rs only contains minimal guidance pertaining to blood sampling.
https://www.rspca.org.uk/documents/1494935/9042554/RSPCA+welfare+standards+for+sheep+%28P DF+10.3MB%29.pdf/e91f2d1e-4a04-30cd-5ed8-8f55da4513c6?t=1594889570996

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed of advances in 3Rs via the Home Office Liaison, as well as via the NC3Rs website and attending relevant seminars/talks. Wherever we find an opportunity to improve our technique/experimental design to minimise animal numbers and/or suffering, we will rapidly incorporate it into our protocols. We closely work with the BSUs at the establishments to discuss how we can improve our animal care, which ultimately improves our results.

175. Improving ruminant livestock production efficiency, quality, health 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
    - 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)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

animal welfare, product quality, environmental protection, nutrients use efficiency, livestock production

Animal types	Life stages
Cattle	juvenile, adult, pregnant
Animal types	Life stages
Sheep	juvenile, adult, pregnant
Goats	juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project seeks to examine ways of optimising efficiency of ruminant meat production from predominately forage-based systems and the associated health and welfare of the livestock. It will examine methods of enhancing product quality, particularly in terms of healthiness, shelf life, hygienic quality, flavour, at the same time as reducing the impact of

these systems on the environment and ensuring farm-based strategies improve animal health and welfare.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Livestock farming, and particularly ruminants (sheep and cattle), is a major component of rural landscapes providing vital services to society through high quality nutrition, soil health, land management for biodiversity and leisure activities and supporting rural communities. But it also polarises society, more so than ever, with debate over animal welfare, impact of animal product consumption on human health and on the environmental footprint of livestock production. This debate is central to the sustainability of livestock farming systems with economic (e.g. production efficiency), environmental (e.g. methane emissions) and social (e.g. animal welfare) parameters and trade-offs widely used to define sustainability. With this project, we aim to address ways of making livestock farming more sustainable in all dimensions to contribute to the "net zero" target for 2050 and with healthier animals and more efficient farms.

What outputs do you think you will see at the end of this project?

Product quality

The research will contribute towards the production of healthier food in terms of a leaner product and which will contain lower quantities of saturated fatty acids and increased content of specific beneficial compounds (i.e. minerals, vitamins and n-3 PUFA) in meat. The role of meat in particular as a vehicle to deliver beneficial minerals, vitamins and fatty acids through to humans. Improved knowledge of the interrelationships between growth of an animal, its production conditions (e.g. grazing system) and subsequent effects on the eating quality of the meat (for example taste and colour) will improve the quality of the product, which is of benefit to the producer and the consumer.

Production efficiency and reduction of greenhouse gases

This work will contribute directly to the overall objective of reducing the output of waste (potential pollutants such as greenhouse gases) per unit of useful product.

Health and welfare

Improving our understanding of livestock systems, including animal health and welfare, has the potential to inform the development of best practice methods of farming. Methods that find synergies between health/welfare, economics, the environment, and society. Regular health and welfare checks will provide insight into risk factors across different systems, animals, and grazing management strategies.

Over the five-year period of the licence, we would envisage the production of 2-3 scientific abstracts to be presented at national and international conferences per year which in turn would be submitted to high impact journals with a total of 10-12 during the life of the project. We envisage the development of 2-3 specific product quality projects related to this licence, 3-4 on production efficiency and 1-2 on health/welfare which would, in turn,

have a pathway to impact for implementation by the farming community – such as decision support tools. We would hope to see these developed during the life of the licence, but some may take longer to implement.

Who or what will benefit from these outputs, and how?

Currently animal agriculture is perceived as inefficient and wasteful in terms of land and nutrient use, this programme of work will improve the efficiency of animal production systems and the quality of the final product. The outputs of this project are targeted and relevant at various levels of the food chain from, the farmer producer, to the meat processor, the retailer and the consumer. In particular, the improved ability to (1) feed ruminants under high forage input systems or sustainable protein systems (2) predict nutrient supply and (3) to manipulate production response will lead to improvements in agricultural efficiency both in terms of use of natural resources and in farm profitability (4) systems can be developed which will reduce incidence of animal disease and associated food pathogens. This will contribute to a reduced reliance on imported feeds, particularly imported protein sources, which is especially prudent following the recent and on-going problems in our industry. Improved knowledge of the interrelationships between growth of an animal and subsequent effects on eating quality (for example tenderness) will improve the quality of the product, which is of benefit to the producer and the consumer. This work will be also particularly important to (but not limited to) ruminant nutritionists (in academia and the livestock industry), agricultural and environmental research scientists, microbiologists and policy makers.

How will you look to maximise the outputs of this work?

The new knowledge generated as a result of this work will be disseminated to the scientific community by publication in peer-reviewed journals and presentation at national and/or international meetings. This will be disseminated to the funding agents, livestock bodies and major meat processors and retailers. We are part of a global network of research farms and we disseminate our findings in international workshops, webinars and conferences.

Data once published in high impact journals will be reported to funding agents to be used in future accreditation schemes and to develop farm-based interventions to improve the quality of meat, production efficiency and animal health/welfare– this could be in the form of decision support tools for the farming community. In particular, the improved ability to (1) determine sustainable supplies of protein to livestock industries, (2) predict nutrient supply and (3) to manipulate production response will lead to improvements in agricultural efficiency both in terms of use of natural resources and in farm profitability. This will contribute to a reduced reliance on imported feeds, particularly imported protein sources, which is especially prudent following the emission targets for 2050. Dissemination to the farming community will occur through interactions at farming events and shows, and through direct interactions with farmers through technology interaction events and farm visits. The translation of scientific results into practical on-farm applications is a key part of the work to be carried out.

Species and numbers of animals expected to be used

- Cattle: 1000
- Sheep: 600
- Goats: 60

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For some of this work there is a requirement to measure various combinations of feed intake, rumen fermentation parameters, product quality, animal health and welfare indicators, and nutrients partitioning between productive (growth and product) and excretion (urine and faeces) purposes. These data can only be collected from live animals, and for the purposes of measuring parameters in relation to meat production there is a clear need to use ruminant livestock (cattle, sheep, goat) in the growing and breeding phase.

Typically, what will be done to an animal used in your project?

Typically, (70%) animals under objectives covering growth performance and product quality and production efficiency will receive 3-4 of the steps stated in the protocol, in rarer cases (10%) animals will experience 4-6 of the steps stated in the protocol or in extreme cases (<5%) animals will have greater than 6 steps. Typically, (80%) of the animals under the health and welfare objective will have a greater number of invasive steps from 4-5, and rarer occasion (10%) 6-7 steps.

The steps are as follows:

Altered housing: animals may be single housed or individually penned, housed in individual 'Chamber', or housed on slatted floors, for a maximum of 12 weeks with a minimum of a days rest for every day penned before the animal can be considered for re-penning. Animals will have sight and sound of other animals

Withdrawal of blood by superficial venepuncture – may be from a one-off sampling for DNA extraction to typically one sampling at the beginning of the assessment period and at the end, with some samplings potentially taking place in the middle

Application of electronic ear tags for monitoring location (GPS) and/or behaviour.

Variation in the composition, constituents, quantity or availability of feed, variation in the composition or constituents of water - typically up to 12 weeks of assessment followed by a resting period of at least two weeks

Administration of substances (e.g. digestive markers, gas tracer, additives) in feed, drinking water, bolus, using a dosing gun for boluses or drench (dosing one bolus up to twice a day for up to two weeks, drenching once a day for up to 9 weeks)

Attachment of harness and separation apparatuses for the collection of urine and faeces - typically for short periods of up to 5 days followed by a resting period of at least one week

Attachment of halters/collars for emissions measurement or tracking position - typically halters for emissions measurement will be attached for periods of up 2-3 weeks followed by a resting period of at least two weeks; collars with GPS sensors can be attached for up to 8 months (during the grazing season).

Manual grab sampling of faeces from the rectum - a maximum of twice a day for up to five days, followed by a resting period of four weeks

What are the expected impacts and/or adverse effects for the animals during your project?

Altered housing: animals may not adapt to the altered housing and may suffer acute stress in the short term until they adapt to the new environment (up to 100% of the animals). Animals may show signs of distress (up to 100% of animals) or stiffness from the slatted floor of the refinement pens (<5% of animals), temporary foot problems or pressure sores from hard floors (<5% of animals).

Withdrawal of blood by superficial venepuncture: in rare cases haematoma formation may occur (<1%).

Application of electronic ear tags: in rare cases haematoma formation may occur (<1%).

Variation in the composition, constituents, quantity or availability of feed, variation in the composition or constituents of water: animals may lose weight (>10% BW) and fitness (<1%).

Administration of substances (e.g. digestive markers or additives) in feed, drinking water, bolus, via dosing gun or drench: animals may show signs of metabolic disorders as a result of the substances (<1%).

Attachment of harness and separation apparatus for the collection of urine and faeces: animals will initially show signs of distress and discomfort (100%).

Attachment of halters/collars for emissions measurement or tracing position: animals getting sores/infection where poor fitting halter/collar causes abrasion of the skin (<5%).

Manual grab sampling of faeces from the rectum: damage to the anal sphincter may occur on rare occasions (<1%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of the animals will experience little more than standard farming procedures. All procedures are not expected to exceed mild in severity (e.g. blood sampling, nasal swabbing and faecal grab sampling). At the end of the trial animals will be signed off the Animals Act before being returned to flocks/herds or sent for commercial slaughter.

What will happen to animals at the end of this project?

- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For some of this work there is a requirement to measure various combinations of feed intake, rumen fermentation parameters, product quality, animal health and welfare and mineral (particularly N) partitioning between productive (growth and product) and excretion (urine and faeces) purposes. These data can only be collected from live animals, and for the purposes of measuring parameters in relation to meat production there is a clear need to use ruminant livestock.

Which non-animal alternatives did you consider for use in this project?

For other areas of work in relation to ruminants for screening additives for their impact on rumen metabolism or pathogens or digestibility of feeds, in vitro measurements of rumen function will be carried. In vitro rumen function studies are carried out using gas production [Theodorou et al., 1994] and rumen simulation techniques [Czerkawski and Breckenridge, 1977]. Much of this work is being carried out as a direct replacement for use of live animals with rumen fluid obtained from an abattoir. Therefore, the use of in vitro analysis replaces the need for greater numbers of animals for direct measurements of rumen function.

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?

Based on the number of cattle used in the Project License no P592D2677 (just below 1000 animals) I consider a similar number will be required for the next five years. For the sheep, I have considered an average of 120 sheep per year which is aligned with the capacity we have in our Small Ruminant Facility (SRF) for detailed intake and nutrients use efficiency studies (three or four trials a year of 24 sheep each in the BioControl pens) with some sheep being assessed outdoor with gas tracers and halters for measuring methane. For goats I have used the maximum capacity of the SRF for hosting goat in one year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We operate standard quality assurance procedures (i.e. the BBSRC/Defra/FSA Joint Code of Practice) and we have incorporated PREPARE and ARRIVE guidelines for preparing and reporting research activities. Statistical advice is sought (from statisticians) on all experimental protocols to ensure that maximum information is obtained from the minimum

resource. An experimental protocol is prepared for each experiment as part of good laboratory practice, and amongst other information each protocol includes:

a statement of objective(s) a description of the experimental procedures, covering matters such as the number of animals required for the experiment, a description of experimental treatments, and the arrangement of treatment allocations (statistical design)

an outline of how data will be collected, and an indication of the 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 measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Some experiments will comprise a combination of in vivo and in vitro techniques to enable more detailed studies of rumen fermentation to be carried out without the use of animals beyond the provision of rumen fluid. In vitro work is essential when using genetically modified plant material, because this cannot be fed to animals due to legislation and because the small amounts of plant material typically available for study are most suited to in vitro study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For much of the work to be carried the effects of treatments on feed intake, production, animal health and welfare, composition, and/or nitrogen and methane excretion in/from specific animal groups (e.g. sheep or cattle) is required. The use of specific breeds will be governed by current practices in the UK so that the results represent current farming. Therefore, these animals are most suitable and refined for use in this work. Before selecting specific animals for trial work their temperament will be assessed as noted by farm staff to ensure the most appropriate individuals are selected to minimise distress whilst on trial.

Why can’t you use animals that are less sentient?

For some of this work there is a requirement to measure various combinations of feed intake, rumen fermentation parameters, product quality, animal health and welfare and nutrients partitioning between productive (growth and product) and excretion (urine and faeces) purposes. These data can only be collected from live animals, and for the purposes of measuring parameters in relation to meat production there is a clear need to use ruminant livestock.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As soon as the animals arrive on site and have acclimatised to their new environment, they will be frequently handled to minimise any stress caused by sampling during the experiment. The handling facilities, weighing platform and crush to be used for the procedural interventions, e.g. blood sampling, faecal grab sampling, bolusing, are close to the animals' housing or where they will be grazing so minimal stress will be incurred moving animals. The animals will be acclimatised to going through the handling facilities and crush without anything occurring to minimise stress when procedures are actually carried out. The cattle and sheep will be trained to halters and any other piece of equipment fitted to the animals (e.g canisters, tubing, GPS sensors) for the purpose of measuring greenhouse gas emissions and tracking location.

All sampling will be conducted on as few occasions as possible consistent with the scientific objectives of the project. Animals will be assessed daily for health and well-being, as determined by alertness, feed and water intake. Any sign of ill health will be reported to the Veterinary surgeon with the animal being removed from trial if symptoms persist and appropriate treatment provided.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow NC3Rs, PREPARE and ARRIVE guidelines.

Also, Norecopa maintains a "Refinement Wiki" (https://wiki.norecopa.no/index.php/Main_Page) with contributions from scientists and animal carers worldwide, which provides a platform where methods for the refinement of animal experiments can be published. This webpage will provide up to date refinement options.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will periodically check the relevant websites and platforms supporting and promoting the "3Rs" (e.g. https://nc3rs.org.uk). I will keep the relevant guidelines ARRIVE and PREPARE at hand and will circulate them among the researchers and technicians taking part of the project.

I will make all the staff involved in the research activities carried out under this project of the latest guidelines and recommendations issued by the relevant sources (e.g. RSPCA, Home Office, Royal Society of Biology, NC3RS). All the research protocols designed for any specific experiment will have a section of Reduction, Replacement and Refinement to be assessed by the PPL, PIL, NACWO, NVS and the Statistician.

176. Crosstalk between inflammation, cell death and cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cell death, autoimmunity, inflammation, cancer, therapy

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate how small messenger molecules called cytokines and immune signalling pathways are contributing to inflammatory diseases and tumorigenesis and how inhibitors affect these 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?

Cell death and inflammation are important processes which are constantly happening in our body. Yet, they need to be well regulated and require a tight control so that their beneficial effect is not turning into a maleficent happening. Upon infections or mechanical damage such as injury of skin or organs our immune system reacts by causing an inflammation and immune response against that damage and the tissue repair system will

eventually restore normal conditions after the healing process is accomplished. Cell death which can occur either unwanted as a negative side effect in an organ/skin injury scenario or as a wanted process to kill e.g., viral infected cells, plays an important role in this process. Too much cell death or an overshooting immune response in our body can result in a reaction against itself and thus can cause a variety of diseases such as autoimmunity, autoinflammation and cancer. Still, the mechanisms leading to these deregulated events mentioned above are poorly understood. In order to develop new drugs for these diseases, it is essential to understand their origin i.e., what is causing the deregulation of events and to characterise the key components involved in this, their activities and how they interplay. To this end we will study how inflammatory and immune pathways correlate with cell death and genes controlling the formation of cancer. In addition, we intend to evaluate the effect of potential inhibitors for specific proteins or activities within a protein that are key for the regulation of such pathways.

What outputs do you think you will see at the end of this project?

The major benefit of this project is that the gain of knowledge on the involvement of tumour necrosis factor (TNF) and other cytokines and immune receptor signalling in the development and/or treatment of cancer and auto-immune diseases such as Crohn's Disease (CD), psoriasis or Rheumatoid Arthritis (RA), and immunity to infections is likely to result in the development of new therapies for cancer as well as chronic inflammatory, auto-immune, and infectious diseases. This project will lead to high impact factor scientific publications that will directly impact the basic research field of immunity, inflammation and cancer by providing mechanistic insight into the molecular pathways regulating key physiological functions such as cell death, survival and associated inflammation.

Moreover, findings of this project will provide fundamental insights for drug development and will consequently impact the clinical field in the long term. Furthermore, these findings could be used diagnostically to predict whether certain immune stimuli, cytokines, immune receptors, or molecules involved in their signalling pathways should be blocked or whether they should instead be employed together with other drugs to reach beneficial therapeutic effects in the above-mentioned diseases.

Overall, the study of genetically modified mouse models with defined oncogenic and/or immune signalling mutations is of great importance for medical research as findings from this project could be harnessed therapeutically in the future.

Who or what will benefit from these outputs, and how?

In order to prevent or reverse life threatening diseases such as chronic inflammation, autoimmunity and cancer new therapeutic treatments are needed. The use of genetic mouse models is crucial to understand these pathways and to test potential inhibitors that could increase human and animal welfare and even be lifesaving.

How will you look to maximise the outputs of this work?

We will be proactive in reading new literature for any new technologies or refinements to existing procedures/experiments. We will publish any new technologies or refinements to experiments we come across during our work. The studies undertaken under this project licence are often collaborations with people, across the UK, and Worldwide. If new collaboration opportunities arise then we will pursue them if they are the best fit for the experimental outcome. We will disseminate the studies undertaken under this project

licence to the scientific community through collaborations, peer-reviewed publications and talks at conferences.

Species and numbers of animals expected to be used

- Mice: 35000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For all investigational work in this project mice are the species of choice. This is due to the fact that the murine immune system and the different modes of cell death happening in mice are well-studied and many features are comparable to the human system. Furthermore, numerous well-defined models for all of the diseases we are aiming to investigate in this project are available. Using genetically altered (transgenic) mouse strains is currently the most effective way to investigate a specific gene function at the level of the whole animal. The application of commercially available products (specific inhibitors for different proteins, small molecules etc.) in our transgenic mice will allow us to investigate the murine immune system and its reaction/response at the most refined level currently possible and to translate our results into the human system.

Typically, what will be done to an animal used in your project?

Mice that have inducible genes that can be switched on or off, will be given inducers such as tamoxifen, either by topically, intravenous or subcutaneous, by gavage or drinking water, that enables gene expression control. Typically, this will be done once, up to several times, over consecutive days, before mice are engaged in the relevant experiment.

Therapeutic substances will typically be administered through routes such as oral gavage, injection or in the food or drink as required.

During survival studies, mice, including those that develop cancer, will be kept for their entire lifespan; that is, up until the humane endpoint - for example, maximum tumour size or the limit of acceptable deterioration of health through observable body scoring. Therapeutics may be given through routes such as oral gavage, injection or in the food or drink and at specific time points mice will be culled by schedule 1 or nonrecovery anaesthesia so that blood and tissue samples can be collected for examination. These can then be processed and analysed to determine whether genetic alteration or administered therapeutics have had any effect on the progression of the disease. Blood samples may also be taken periodically from these mice for pharmacokinetic studies.

Immune-deficient mice may also be injected with cancer cells, for example subcutaneously, and treated with therapeutics. These studies generally last 2 to 3 months and allow us to study the treatment and progression of cancers and the role of the immune system in cancer.
Mice that undergo a surgical procedure, are expected to make a rapid and unremarkable recovery from surgery, within 48hrs. Mice will be given analgesics to alleviate any pain

prior and post-surgery to aid recovery. Surgery will be performed only once unless an animal needs to be re-sutured.

Mice will also undergo relevant imaging modalities depending on the need they may undergo several imaging sessions. This means they will undergo several rounds of anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Weight loss - gradual over several weeks or more quickly over a few days. Not more than 15% of starting weight within deviation from the start of experiment.

Deterioration in general health caused by progression of disease such as inflammation or cancer as assessed by body scoring. For example, staring coat, laboured breathing and hunchback position that would last for no longer than 24hrs.

Some of the animal models we intend to use spontaneously develop an inflammation of the skin also known as dermatitis. If we are not able to revert this condition by genetic experiments or chemical/biological treatments the animals will be humanely killed as soon as they start to show signs of distress (hunchback position, excessive scratching, etc).

Other animals develop swollen lymph nodes; this condition is not harmful for the animal, and they will be either bred or used in experiments. Animals will be humanely killed as soon as enlarged nodules restrict movement or any other physiological condition. Some other adverse effects may arise by the application of chemical or biological substances into the mice. The animals will be closely monitored during these procedures and will be humanely killed as soon as they start to show signs of distress (hunchback position, lack of grooming, excessive loss weight, etc).

Expected severity categories and the 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 breeding and maintenance of genetically altered mice should generally cause no more than sub- threshold severity in the case of mice that produce phenotypes that have no clinical manifestation. In the case where phenotypes show clinical signs, they are generally mild and in the case of harmful mutants where tumours arise, mice will be culled before the onset of clinical signs such that breeding mice will not experience severity greater than mild or in a rare case moderate.

In the case where mice are injected with inflammatory substances, injected with cancer cells or develop spontaneous phenotypes with clinical manifestation, then mice will suffer no more than moderate signs of distress if the scientific endpoint is reached before the humane endpoint.

We should be able to generate and collect most of our data under a mild severity. However, if further data is required, then mice will be allowed to reach a humane endpoint where they will experience moderate signs of distress for no more than a day or two.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

All of the planned work with mice is generally preceded by intensive studies done in the laboratory with cell lines and organoids. Before carrying out any experiments in the mouse we will make sure to monitor for as many physiological conditions as possible in our various cell lines and organoids. Yet, although the in vitro work can provide important molecular and cell physiological insight it unfortunately cannot fully recapitulate the complexity of a pathophysiological situation in the context of inflammation and cancer in a mouse or a human.

Which non-animal alternatives did you consider for use in this project?

All of the planned in vivo work is generally preceded by intensive in vitro studies. Cell lines have been generated from the transgenic mouse strains. These include Mouse Embryonic Fibroblasts (MEF) from knockout mice and tumour-derived cell lines as well as tumour-derived organoids from GA mice engineered to express activated oncogenes and inactivated tumour suppressor genes. Human cancer cell lines and organoids with defined genetic alterations are also present. They will be monitored for migration, proliferation, and apoptosis as well as long term survival. Then, cytokine-of-interest blocking agents as well as RNA interference of particular factors will be tested on the cell lines and organoids. The use of matrigel in transwell assays can simulate extracellular matrix in an invasion assay.

Why were they not suitable?

The use of 2D and 3D cellular systems as well as co-culture with different types of immune cells, fibroblasts and endothelial cells cannot fully recapitulate the pathophysiological situation in the tumour microenvironment. However, it can provide important molecular and cell physiological insight and a justification for further in vivo work. In addition, the evaluation of the morbidity caused by inflammation in vivo is irreplaceable with the in vitro studies since the cell lines do not completely recapitulate the inflammatory circumstances in the 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 numbers of mice we would like to use for the experiments would primarily have been based on previous experiments or those performed in the literature or by collaborators. We have also looked at statistical analysis for numbers of animals in experiments. In this way we are confident to produce meaningful scientific data with the least number of mice possible.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our preceding in vitro experiments are going to limit the number of animals required for the in vivo investigation as key components involved in inflammation and oncogene-driven cancer can be identified in 2D and 3D cell culture systems.

Mouse colonies will be closely managed by members of the group to avoid excessive breeding.

For every in vivo experiment we write a protocol which includes a statement of the objectives, a description of the experiment listing experimental treatments, number of mice per treatment group and the experimental material needed. Further reductions in the number of mice will be achieved by improved study design and statistical methods which will allow us to integrate and aggregate the data across multiple experiments thus achieving a statistical conclusion which makes additional experiments with animals superfluous.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Experiments will be conducted using inbred and gene-targeted mice on a C57BL/6 background, which is the most common and well-described strain. We have planned mouse crossings which are aimed at providing us with mice that carry up to seven different transgenes. In order to receive statistically significant numbers of mice carrying the respective phenotypes we calculated probabilities according to Mendelian ratios. We will cancel the breeding pair once the new litter will give us the mice with desirable genotypes. Long term experiments will be done using sufficiently large groups of mice per phenotype to reach statistically significant results. As the different groups will in some cases be composed of mice from subsequent litters from the same breeding pair, following cumulative analysis of the mice in each consecutive litter we will be able to determine statistical significance following final double-blinded analysis of each litter. Thereby we will perform the experiments using 4-25 mice per experimental group depending on the experimental design, in order to obtain statistically significant results. The data generated from regular monitoring of the mice (primary tumour formation or occurrence of inflammatory lesion, incidence, progression, metastasis formation, clinical score, inflammatory phenotype) will be analysed using the Log rank test on Kaplan-Meier representation. Data generated from tissue analysis will compare mutant mice to wild type littermates and will be evaluated by student t test. The proposed experimental design and methods of analysis of the results will be further discussed in detail with the Statistical Services Unit.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The overall plan of this work is to first, understand what leads to deregulated cell death, inflammation and inappropriate immune responses which can result in autoinflammation, autoimmune diseases and/or cancer and second, to develop novel therapies to benefit human health in targeting these diseases. For all investigational work in this project mice are the species of choice. Furthermore, numerous well-defined models for all of the diseases we are aiming to investigate in this project are available. Using genetically altered (transgenic) mouse strains is currently the most effective way to investigate a specific gene function at the level of the whole animal. The application of commercially available products (specific inhibitors for different proteins, small molecules etc.) in our transgenic mice will allow us to investigate the murine immune system and its reaction/response at the most refined level currently possible and to translate our results into the human system.

All of our procedures are designed to minimise pain to the animal as much as possible. Yet, certain procedures will have a moderate impact on the wellbeing of the animal. Thus, we have developed pre- defined endpoints to pre-empt and avoid the onset of any adverse effects and to keep the negative impact on their welfare as low as possible. These pre-defined endpoints take different parameters into account such as behavioural changes, body weight etc.

In the procedures where we induce tumours for instance the pre-defined endpoint is reached as soon as the tumour burden appears to impair its mobility and the mice show signs of distress such as lack of grooming and separation from the group.

In every procedure we try to keep the length of time for each experiment to a minimum.

Additionally, a close monitoring of animals throughout any kind of treatment or of those which are genetically prone to develop a phenotype will guarantee that they are not suffering excessive adverse effects.

Concerning the mice which are prone to develop diseases due to their genotype (e.g. the mice which develop inflammation of the skin), we have established a breeding strategy which allows us in many cases to delay or completely prevent the onset of disease by combining certain gene mutations thus contributing to the welfare of the animal.

In addition, the institute in which the animal procedures are carried out, runs a comprehensive health- monitoring programme. Animal health and welfare records are maintained to include any adverse effects that may develop, particularly in genetically altered and spontaneous mutant strains. Signs consistently associated with a particular phenotype/genotype will be recorded on the respective “information sheet” in the breeding area and the mice will be maintained under conditions where their health status can be protected as far as it is reasonably practicable.

Why can’t you use animals that are less sentient?

Mice are the species of choice in this project as the murine immune system and the different modes of cell death happening in mice are well-studied and many features are comparable to the human system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For protocols that require surgery, procedures will be carried out aseptically and post-operative analgesia will be given after surgical procedures to manage any pain.

Where the immune status of the animals might compromise health, they will be held in a barrier environment.

During breeding and experimental scenarios arise where mice find themselves individually housed (for example, litters with only one male, if mice need to be culled at different time, according to the humane endpoints points, during experiments). To minimise this from occurring, weaning will be managed to avoid singly housed animals wherever possible and mice that are of no scientific interest or are not required for breeding will be culled. In cases where singly housed males are to be kept for experimental or breeding purposes, post productive females will be sought as cage companions.

Breeding calculations will be used to maximise breeding schedules and minimize the number of animals needed for maintenance and experiments.

If protocols require anaesthesia, this will involve agents and methods suitable for the species as advised by the NVS. Depending on the anaesthetic regime used, animals will be ambulatory within a few minutes to 1hr of the end of anaesthesia. Supplemental heat and analgesia will be provided as advised by the NVS during surgery and recovery. Any animal which has not resumed normal mobility, eating and drinking by the end of the working day of surgery will be killed.

Mice will be observed to monitor the onset of any phenotype; weight, piloerection, abnormal breathing rate, skin turgor, behaviour, eye discharge and mobility will be checked as indicators of suffering. Any deviations from the norm, including the timing of the onset of suffering, will be monitored and used to predict future occurrences. If sufficient scientific data is obtained before the humane end point of the experiment is reached mice will be culled to prevent further suffering.

Suitable administered volumes and doses of therapeutics, immunogenic substances or cells will be determined by referring to our own, collaborators and published data. If suitable doses and volumes are not known, then pilot studies will be initially carried out. Pilot studies will consist of injecting one animal with a low dose and increasing the dose stepwise in other animals, until the desired scientific endpoint is reached, or before a moderate degree of suffering is reached, whichever is lowest – this will dictate the maximum dose for the experiment. The effects of each dose will be evaluated before adopting a stepwise approach to higher doses, such that the humane endpoints are not breached.

During cancer studies, animals will be observed for signs of tumour growth for their entire lifespan by blood sampling and calliper measurements of external tumours. For animals with internal tumours experiments will be carried out with routine necropsies taken to determine the growth rate of the tumours and its effect on the welfare of the animals. This will enable the timing of scientific and humane endpoints to be determined.

Other than in terminally anaesthetised animals, dosing and sampling procedures will be undertaken by a person competent in the relevant technique, in accordance with good practice using a combination of volumes, routes and frequencies that will result in no more than transient discomfort and no lasting harm and are the minimum compatible with the scientific objectives.

The latest procedures that cause the least suffering to mice will be used to replace older, outdated methods. Unless otherwise stated in the protocol. Ongoing training will be given in new and existing procedures to ensure best practice is occurring.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best guidance practice published on websites such as NC3Rs and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By literature searches and staying up to date with the NC3rs website and attending NC3rs symposiums. Checking with the NVS, NACWO and BSU staff before any procedure is carried so that we can implement these techniques effectively. Collaborating with researchers who have carried out this work before.

177. Role of blood vessels in bone 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

blood vessels, bone, ageing, haematopoiesis, microenvironment

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how blood vessels inside the bone help to regulate specific microenvironments that contribute to bone physiology during growth and ageing. We want to do this so we can develop new strategies to improve bone health in the ageing population.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Age-related bone loss and other bone conditions, such as osteoporosis and delayed fracture repair, are extremely prevalent among the ageing population. With increasing life expectancy and lifestyle and demography changes, it is important to tackle

musculoskeletal conditions for healthy ageing. In addition to bone loss and fracture risk, re-establishment of healthy bone marrow in patients following chemotherapy or radiotherapy is crucial to maximise the treatment outcome of bone marrow (BM) transplantations.

The bone interacts with other organs in the body to tightly regulate whole-body health. Changes in bone mass are linked to ageing and diseases such as diabetes, hypothyroidism etc. These changes involve modifications in smaller localised 'microenvironments' within the bone. Blood vessels in the bone play a key role in regulating these microenvironments, and are important for supporting these microenvironments for the healthy formation of bone and production of blood cells within the bone marrow. Despite their importance, bone blood vessels and their microenvironments have not been well- understood. In this programme, we investigate blood vessels and their specialised microenvironments to explore their functions in bone health, repair and disease, and to develop new therapeutic strategies for managing bone and blood diseases in the future.

What outputs do you think you will see at the end of this project?

The ultimate aim of this project is to understand blood vessel functions in skeletal physiology. We hope to better understand how blood vessels in the bone interact with other cells in the bone to affect bone formation and blood cell production. Importantly, our findings in this project could identify new strategies which target blood vessels to treat patients with bone and blood diseases.

This research will generate data which will be published in peer-reviewed journals, contributing to the advancement of knowledge in this field. We plan to publish this research in the highest quality journals (where it is well-cited and influential) and at international conferences, ensuring that it reaches the widest possible audience. Our data on bone health and diseases will also be communicated to the public through accessible presentations and innovative workshop formats.

Who or what will benefit from these outputs, and how?

My team will constantly discuss our findings with collaborators and the UK scientific community from the beginning of this project. We will disseminate important findings and techniques which are beneficial to other labs around the world who are carrying out research in the field. We will discuss our findings and observations with clinicians, to maximise the potential of our work to be applied to help patients with bone diseases in the future.

We believe new suitable pharmacological targets for treatment of bone conditions would be proposed in the next five years. These findings would potentially benefit clinics in the next 10-15 years. We will also constantly interact with the public through presentations and workshops which would benefit the public by providing essential knowledge on lifestyle for healthy living.

How will you look to maximise the outputs of this work?

My team and myself will be constantly involved in the dissemination of the findings to the scientific community and will also make these findings accessible to non-specialized audiences every year through public engagement activities. In addition to research collaborations, I am establishing clinical collaborations to take our findings to clinics, which

will maximise the development of effective therapeutics for patients. We are also interacting with industrial partners to design/screen drugs to target specific cell types or functions. We will present our findings in scientific meetings, discuss the benefits with the public and publish the work in high impact journals to disseminate the knowledge and maximise the outputs of this work.

Species and numbers of animals expected to be used

- Mice: 12500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As this research investigates blood vessels and the skeletal system in the context of whole-body physiology, the use of a mammalian model system is a fundamental requisite. Importantly, the unavailability of an artificial model system mimicking human blood vessel functions is the primary motivation to perform this study on animal models. Mus Musculus (laboratory mouse) is the most commonly used mammalian model system. The mouse genome is sequenced, and it shows similarity to the human genome. The relevance of genetic and physiological context to the human system makes it the appropriate model system to investigate systemic physiology. In particular, mice have a skeletal system that looks, ages and develops diseases that resemble humans. Thus, the physiological interaction of the cardiovascular and skeletal systems in health and disease can be efficiently studied using mouse models. In addition to being the smallest mammalian model, the availability of a wide variety of genetic models, short reproduction cycles, and low maintenance costs compared to other models make the mouse the best mammalian model system available. Moreover, it is a powerful system to manipulate genes in a tissue-specific and time-dependent manner to study cell-specific functions and understand age-associated molecular mechanisms.

Most of our experiments use the postnatal development stage to understand the mechanisms that regulate anabolic mechanisms in the bone. We then use these findings at this stage to manipulate aged and diseased mouse models.

Typically, what will be done to an animal used in your project?

Typically, we target blood vessels using various strategies and disease models to understand their functions. We breed and generate genetically altered (GA) mice which will then be administered with inducible substances orally or injections usually five consecutive days to induce genetic changes. The mice will be humanely killed at the end of the experiment usually after 2 weeks to study functions of genes. Similarly, we also use wildtype mice to administer substances (dosage and route depending on the substance) to induce disease models or to inhibit blood vessels. Sometimes both wildtype and GA mice are allowed to age (>1 year) and performed genetic alterations by inducible manner (as explained earlier) to study age-related changes in the bone. Mice will be humanely killed at the end of the procedure to determine changes in bone, circulating blood and blood vessels.

We also use GA mice to visualise blood vessels in a living mouse. Mice will be anaesthetised and maintained under continuous anaesthesia during imaging process. Mice will be humanely killed at the end of the procedure without regaining consciousness.

What are the expected impacts and/or adverse effects for the animals during your project?

Although no adverse effect is expected after genetic manipulation, deletion of some genes may result in unexpected traits. In this case, induced mice will be closely monitored and the termination time of the experiment will be brought forward as required. Therefore, mice will be carefully monitored for adverse effects and will be killed if the condition is prolonged for more than 24 hours. In general, we will always seek advice from the local Named Animal Care and Welfare Office (NAWCO) and Named Veterinary Surgeon (NVS) if any mouse shows adverse changes.

In ageing experiments, mice older than 15 months will be closely monitored for the severity of age- related symptoms. Generally, mice will be used before they reach 18 months of age.

In general, if the administration of any pharmacological drugs or their solutions causes local rashes/irritation/inflammation/infection or any unexpected adversities, we will immediately seek the advice of NVS and NACWOs to manage those animals and future experiments.

Whole-body irradiation damages primarily cells dividing at a high rate such as blood cells and gut cells. Adverse effects of whole-body irradiation are noticed in less than 1% of animals undergoing the procedure. Irradiation commonly affects pigment production leading to dark furred mice producing grey/white coats which has no adverse effects on the animal.

To minimise the presence of potentially pathogenic agents, mice of the cleanest available health status are used, held within clean housing conditions (such as isolator, filter cages, clean enclosure rooms or at least maximal hygienic measures) at all times. Any mouse receiving a single dose of radioactivity will be maintained on antibiotic treatment for at least 5 weeks after the last irradiation dose to prevent diarrhoea or weight loss possibly arising through cells damage in the intestines. Any animal showing abrupt weight loss of pre-irradiation body weight, and/or other signs of illness will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the majority of our experiments, the actual level of severity involved in the procedures is mild. Based on our last year record, minimum 80% of mice undergo mild procedures and the maximum of 20% of mice will be in moderate level.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed study investigates the physiology of blood vessels in the skeletal system and their relevance to ageing. Animal models have proven to be more relevant to investigate blood vessels in organ- and whole-body physiology. Importantly, the unavailability of an in-vitro model system mimicking human vascular functions is the primary motivation to perform this study on animal models. Mus musculus (laboratory mouse) is one of the best model systems available to study the mammalian physiology and pathophysiology of blood vessels and the skeletal system. Though some details can be studied on cultured cells, endothelial cells do not represent the way they behave in intact animals, e.g. transport of blood. They do not demonstrate the diversity in function and structure observed in intact animals. There are no cell culture models available that can replicate the system in vitro. Therefore, the use of animal models is inevitable for this programme, which dwells in the systemic understanding of skeletal physiology. Animal models can provide systemic insights into the ageing process that cannot be addressed by in vitro model systems. Moreover, the experimental findings from this animal work can be generalised and beneficial to understanding human physiology and disease.

Which non-animal alternatives did you consider for use in this project?

Where possible, we make use of cultured cell lines to carry out pilot experiments to gain some information about the behaviour of gene constructs before hypotheses are tested in mice. In addition, culturing of cells isolated from genetically altered mice constitute replacement as they replace procedures that would otherwise be carried out in mice. To note, we are also developing a novel three-dimensional culture system to replicate a bone microenvironment in a dish. If we establish this method, it would reduce the number of mice used in bone research not only in our lab, around the world.

Why were they not suitable?

In vitro systems cannot provide systemic insights into blood vessel functions and ageing of the skeletal system. In general, the basic research is at its initial stages of understanding blood vessels formation and structure. We are still unable to generate functional blood vessels of a particular organ system in vitro. Therefore, we do not have an alternative system to study vascular function in skeletal 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?

The number of mice was estimated considering the scientific objectives outlined in the programme of work and anticipated numbers of experiments and mice. The estimates are based on similar studies conducted in the past using similar protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

At every stage in our experiments, consideration will be given to ways in which we can reduce the number of animals. Several of the protocols that we use are designed in such a way as to obtain the maximum possible data from a single animal. In my team, all animal experiments are planned and designed to reduce animal usage and increase maximum output. We use tissues from a single animal for multiple analyses that involve advanced cellular and molecular techniques. This is evident from the current animal use data in my project license.

Genetically altered (GA) mouse models will be generated by the highly experienced staff of our dedicated transgenic facility. Best practice will be followed but it is difficult to predict how many animals will be required as this depends on the expression profile of different constructs. Where GA animals are generated in house, established breeding practices will be followed that are designed to generate the minimum number of animals.

We will consult with statistical experts within the institute. We will use appropriate statistical tools to assist in planning or designing experiments to calculate the number of mice needed to generate meaningful data. Otherwise, we will use the least number of animals to provide an adequate description, generally on the basis of previous experience. In terms of the numbers of animals required, usually 6-10 animals per treatment group are sufficient to obtain the required results. In addition, we will use the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines issued by National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) in reporting our results, and in considering the design of our experiments (e.g., allocation of animals to groups, blinding, etc).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Sharing of tissues is well established in my team; every team member coordinates with others in best and maximum use of every single mouse in our experiments for e.g. we use genotypically unsuitable mice from our breedings for in vitro studies.

Our expertise and more than a decade of experience in transgenic mice allow us to efficiently reduce unnecessary mouse breedings for our experiments. Mice sharing strategy in our lab permits efficient use of genetically unsuitable mice from the breedings.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All the procedures in this licence are classified as either mild or moderate and are done under local or general anaesthesia, where appropriate, to minimise stress and suffering of the animals.

We will use wildtype and genetic altered mouse models to induce changes in the blood vessels and bone, to understand the mechanisms involved in bone physiology. To study gene functions, we use inducible cell-specific mouse models which do not induce genetic changes in the whole body reducing the harm and stress experienced by the animals. Scientifically, it will allow us to study cell specific functions of a gene without altering other cell types. We will also administer pharmacological substances to target particular molecular pathways in the bone. There is the potential for suffering when substances are administered to conscious animals. Animal suffering is minimised by following careful aseptic techniques and handling by skilled scientists. In general, these genetic alterations and substances are not expected to cause distress or lasting harm to the animals. We will administer the minimum doses required and minimise the length of experimental conditions (most lasting 2 weeks or less), to minimise stress and inflict the minimum pain.

Why can’t you use animals that are less sentient?

We need to use mice because we need to work with mammals since they have bones, blood and a circulatory system that function similar to humans’. Non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) or less sentient animals (e.g. zebrafish) have different physiologies, so cannot be used for this study. We also need to work with adults and ageing mice because we are studying how and why ageing affects bones and blood vessels.

Mice are the simplest and most humane mammalian model we can use to study bone physiology. Because all mammals are sentient, we follow methods to reduce distress, pain, suffering or any harm to mice in our experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In general, we provide extra care and closely monitor the experimental mice to minimise any welfare issues. If any unexpected signs of distress are observed, we will stop the experiment early and humanely kill the affected animal. We will try to make all animals as comfortable as possible, whether or not they are undergoing experimental procedures, e.g. by providing an enriched environment and careful handling to minismise stress. We will get mice used to people and handling from a young age, to reduce their stress levels during protocols where they may be handled frequently. When appropriate, analgesia will be provided.

To administer pharmacological treatments, and especially when medium-long term treatment is needed, multiple injections will be replaced by osmotic minipumps, whenever possible. Dose-response and time course analysis for induction of gene manipulations will be performed to maximise research outcomes, but with a minimum consequence to the animal’s health. Animals will be kept warm and monitored regularly during and after anaesthesia. We will regularly consult with the NACWOs, NVS and colleagues about best practices and potential further refinement of our procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the guidelines mentioned in Animal Research: Reporting In Vivo Experiments (ARRIVE) and Laboratory Animal Science Association (LASA). We also refer to LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery, and Laboratory Animals Limited (LAL) for progressing the care, husbandry and management of ageing mice used in scientific studies.

For best practices in severity limits, we take guidance from the following published literature:

Jones HRP, Oates J, Trussell BA: An applied approach to the assessment of severity. In: Humane endpoints in animal experiments for biomedical research. Proceedings of the international conference, 22-25 November 1998, Zeist, The Netherlands, edited by CFM Hendriksen and DBMorton, pp. 40-47, 1999.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I and my team will be in regular contact with the local facility technicians, NACWOs and NVS to review current approaches and any new opportunities in the Replacement, Refinement and Reduction of animals in research. We also follow NC3Rs newsletter to get information on recent advances, opportunities and events. We will follow NC3R events, symposia and events to get more information about tools and technologies at the other institutes.

178. Embryonic origins of 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

Key words
embryonic, cardiovascular, heart, circulation, development

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine some of the mechanisms via which adverse conditions during prenatal development trigger embryonic origins of 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?

To diminish the burden of cardiovascular disease by understanding mechanisms better in order to identify plausible therapies for intervention

What outputs do you think you will see at the end of this project?

The outputs that we will see at the end of the project will be:

Data relating to cardiovascular function, cardiovascular morphology (structure) and underlying molecular pathways in chicken embryos incubated under control of challenged

conditions (e.g. lower than normal oxygenation or hypoxia) with and without treatment (e.g. antioxidants);

Data relating to cardiovascular function, cardiovascular morphology and underlying molecular pathways in hatchlings raised from fertilised eggs incubated under control of challenged conditions (e.g. hypoxia) with and without treatment (e.g. antioxidants);

Data relating to cardiovascular function, cardiovascular morphology and underlying molecular pathways in juvenile/adult birds raised from fertilised eggs incubated under control of challenged conditions (e.g. hypoxia) with and without treatment (e.g. antioxidants);

Data relating to cardiovascular function, cardiovascular morphology and underlying molecular pathways in first and second generation birds raised from fertilised eggs incubated under control of challenged conditions (e.g. hypoxia) with and without treatment (e.g. antioxidants).

Publications as abstract, papers, reviews and book chapters of Points 1-4;

Dissemination of unsuccessful approaches or negative findings.

Who or what will benefit from these outputs, and how?

The work in this project licence is to be done in the chicken. The reason is because in contrast to all mammals (except primitive mammals that lay eggs or monotremes), the chicken permits some of the direct effects of adverse conditions during development on the embryo to be isolated, independent of effects on the mother and/or the placenta. Establishing direct effects on the developing heart and circulation means we can better identify mechanisms involved. This will improve intervention to guide future translation into human clinical practice.

In the short-term term (1-3 years), the data outputs of this project licence (e.g. changes in arterial blood pressure, heart rate, cardiac function) will inform us how adverse conditions during early life may increase the risk of cardiovascular dysfunction later on in the adult offspring. For instance, is the heart affected more than the vasculature? Which is worse and which may contribute more to triggering cardiovascular disease?

In the medium-term (3-5 years), the effects of the experiments in chickens will also inform our programmes of work and those of other researchers in other species, such as in the mouse, rat and the sheep. It will tell us which are the areas to focus on, and what similarities and differences there are between species. The idea is then to create a layered approach of understanding on the effects of adverse conditions during development on the cardiovascular system across the life course in different species. This will help to better translate our findings to the human clinical situation. For example, knowledge of common effects of an adverse condition in early life on the heart of the chicken, mouse and sheep may precipitate human benefit by designing a treatment with protection across species and thereby likely to be as efficient in humans.

How will you look to maximise the outputs of this work?
The outputs of this work, including the dissemination of unsuccessful approaches or findings, will be maximised at several levels:

Scientific advancement and collaboration: In the longer term (>4 years), the data will benefit the design of therapies in higher vertebrate models of adverse pregnancy with a view to human translation and the design of clinical trials. This will be achieved via collaboration with experts in different fields within and outside the university. Therefore, the proposed work in this new project licence may hasten translation to relatively simple but novel human clinical interventions to not only treat the mother, but also her progeny. This will contribute to a reduction in the burden of developmental origins of heart disease, thereby having a positive clinical, economic and societal impact on health.

Dissemination of new knowledge. Other pathways to further increase impact will include contacting the funders and the University communications office to alert them of the potential influence for human health of the scientific findings. This will lead to press releases, which will be supported by radio and television interviews. In addition, the data will benefit the design of cures to protect the health of the unborn child. The proposed research is therefore likely to be of significant interest and benefit not only to researchers carrying out similar or related research in the field, but also to national and international researchers in other disciplines, such as biochemistry, pharmacology and nanotechnology, as well as cross-disciplinary teams in the pharmaceutical industry. To deliver translational benefit to the nation’s health, wealth and culture we will adopt a number of strategies such as seeking patent protection for any new therapies or diagnostic biomarkers revealed by the research as well as actively engage with the commercial pharmaceutical and healthcare sectors to exploit our research at the earliest opportunity.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The chicken is the ideal animal for the project as it permits isolation of the direct effects of adverse conditions during development on the cardiovascular system, independent of confounding effects of these challenges on the mother and the placenta (as in mammals). This will help us better isolate the direct effects of challenges to development, underlying mechanisms and therefore the improved design of possible cures.

The studies will focus on the chicken embryo, the hatchling and the juvenile/adult bird as the project is designed to determine the effects of adverse conditions on the cardiovascular system of the progeny across the life-course, from embryonic stages through to the adult offspring.

Typically, what will be done to an animal used in your project?

The project focusses on three life-stages of the life-course: the chicken embryo, the hatchling and the juvenile/adult bird.

In the chicken embryo studies, typically a fertilised egg will be exposed to control (e.g. normal air or normoxia) or challenged conditions (e.g. lower than normal oxygenation or

hypoxia) during incubation and the chicken embryo studied in late incubation (e.g. at day 20 out of 21-day incubation period). The majority of the studies will be ex vivo (after death), i.e. in tissues isolated from animals followig Schedule 1 killing. A minority of studies will investigate the function of the heart and circulation under terminal or recoverable anaesthesia following surgery. Under terminal or recoverable anaesthesia, typically 1 experiment will be performed in any one embryo, lasting approximately 5 hours.

In studies in the hatchling, juvenile or adult bird, typically a fertilised egg will be exposed to control (e.g. normoxia) or challenged conditions (e.g. hypoxia) during incubation and the chicken embryo will be allowed to hatch. The majority of the studies in the hatchling (typically 1-2 weeks of age) or in the juvenile or adult bird (typically 12 weeks to 1 year of age) will be ex vivo, i.e. in tissues isolated from animals following Schedule 1 killing. A minority of studies will investigate the function of the heart and circulation under terminal or recoverable anaesthesia following surgery. Under terminal anaesthesia, typically 1 experiment will be performed in any one animal, lasting approximately 5 hours. Under recoverable anaesthesia, typically 3 experiments will be performed in any one animal, with 1 day rest in between. Each experiment will typically last 3 hours.

What are the expected impacts and/or adverse effects for the animals during your project?

It is well established that incubation of fertilised chicken eggs in adverse developmental conditions, such as lower than normal oxygenation or exposure to glucocorticoids (steroids) can reduce embryonic growth and survival. Our own experience is that about 15% of eggs are not fertile (checked by candling) and about 10% of embryos incubated artificially under optimal and normoxic (normal air)
conditions die normally, usually stopping development within the first 5 days of incubation. Exposure to 14% hypoxia (lower than normal oxygenation) between day 1-19 of incubation reduces the survival further by ca. 40% and hatchability by 50%. Relative to embryos incubated under normoxic conditions, surviving embryos of hypoxic incubations show a ca. 25% reduction in body weight. Our experience with glucocorticoid therapy is that treatment of the chicken embryo with synthetic steroid hormones, such as dexamethasone or betamethasone reduces survival by 20% and it decreases fetal body weight by 20% by the end of the incubation period. Therefore, we expect similar adverse effects of incubations with periods of lower than normal oxygenation or treatment with glucocorticoids during the tenure of this project licence. We do not expect other adverse effects of experiments at the chicken embryo stage of the life-course.

We do not expect any adverse effects of topical administration of substances (e.g. antioxidants or vehicle) onto the chorioallantoic membrane (membrane that separates the embryo from the air cell within the egg) between day 1-20 of incubation, via a 1 mm hole drilled into the eggshell. The hole in the egg shell will be covered by a small piece of tape after substance administration. We have plenty of experience with this route of administration and we have not seen problems, such as infection, for instance in 5 years.

Conversely, between day 15-20 of incubation, administration for example of antioxidants, or vehicle via intravenous, intramuscular or intraperitoneal may cause bleeding. Intraperitoneal injection in birds also increases risk from flooding one of the air sacs. If air sacs are compromised and /or bleeding on any one day is estimated to be >10% of the estimated embryonic blood volume, the animal will be killed by a Schedule 1 method. Similarly, if blood loss could not be controlled, the animal will be killed by a Schedule 1 method. Embryonic blood volume can be estimated from comparison of the size of the

embryo to our own historical library of sizes and weights of embryos at different stages of the incubation period.

Following hatching, growth of hatchlings of incubations under adverse conditions may also be slower compared to controls. For instance, our own experience is that the weight gain of hatchlings from hypoxic incubations at 6 months of age is reduced by an average of 1-2 g/day. Therefore, we expect similar adverse effects of incubations under sub-optimal oxygenation on post-hatching growth with or without embryonic and/or post-hatching treatment with glucocorticoids during the tenure of this project licence. Conversely, we expect hatchlings exposed to an obesogenic diet (a diet that makes you gain weight) to put more weight on than controls as they grow to adulthood.

Some protocols require treatment of hatchling/juvenile/adult birds with intramuscular injections (in the muscle). Intramuscular injections in juvenile/adult birds will be given in the pectoral muscle. This may cause transient pain.

Exposure of hatchling/juvenile/adult birds to an obesogenic diet may lead to fatty liver. We do not expect that this will affect the wellbeing or behaviour of the animal during the tenure of the protocol.

While under terminal anaesthesia, some protocols may require surgical implantation of probes or occluders, or the application of vessel occlusion. Some of these procedures may lead to unexpected bleeding. If more than 10% of the estimated blood volume of the animal is lost, then the animal will be killed by a Schedule 1 method.

A minority of experiments in the hatchling/juvenile/adult birds require surgery under recoverable anaesthesia. All surgical procedures will be carried according to the Home Office Minimum Standards for Aseptic Surgery. Through previous work, we have gained significant experience with work on birds following surgery. Pain killers will be administered as required, judging from the animal behaviour. In some animals, arterial catheters will be placed which allow for blood sampling. The health of the bird can then be monitored through measurements of blood gases. For example, we will be able to ask if oxygen levels in blood are normal?

Some protocols require study of the bird after removal of the nerve that is attached to the carotid body (denervation of the carotid bodies) or removal of the adrenal glands. Denervation of the carotid bodies does not produce any resultant harm. In fact, carotid body denervation is currently being trialled in human patients to treat hypertension. In contrast, removal of the adrenal glands can trigger adverse effects on blood volume and blood pressure over prolonged periods of time. Adrenal insufficiency in man and other animals is known as Addison’s disease and these individuals through hormone insufficiency can develop low blood pressure over a period of months. To minimise this possibility, no animal will be studied and kept for longer than 1 month after surgical removal of the adrenal glands.
In the event of post-operative complications, such as a catheter being removed, birds will be killed unless such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence (bursting open), uninfected wounds may be re-closed on one occasion.

We estimate that about 10% of birds will not be able to weight bear on one or both legs after one day's recovery from surgery. In this instance, the animal will be given supportive care via a sling and it will be monitored closely by taking morning and afternoon arterial blood samples. The animal's food intake and ability to pass faeces will also be closely

monitored at least twice daily. Any birds that cannot weight bear on two legs for 72 hours despite continued pain killer care, appropriate food intake, defecation and blood glucose concentration will be killed by a Schedule 1 method or earlier if its condition deteriorates before this point.

For studies involving a scan of the heart in the conscious bird, the plucking of feathers under gentle restraint to expose any area will be minimised. Feathers will be plucked in the direction of their insertion into the skin to avoid skin rupture and discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Species: Chicken Mild: 90%
Moderate: 10%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In some experiments, we must use whole animals or organs isolated from animals because function, for instance in the cardiovascular system, is regulated by complex networks, none of which have been reconstituted completely in computer models. The overall system is not well enough understood to make mathematical modelling useful.

Which non-animal alternatives did you consider for use in this project?

There are no suitable non-animal alternatives to use in this project.

Why were they not suitable?
No non-animal alternatives are suitable to use in this project as none can model the effects of adverse conditions during embryonic development in programming an increased risk of cardiovascular disease in the adult progeny.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals that we will use in this licence from experienced use of animals, as detailed in more than one retrospective review using this species.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental design was created with the NC3R's Experimental Design Assistant to ensure reproducibility using the least number of animals to satisfy statistical power for anyone measurable output.

To ensure the minimum number of animals is used in this project to address all objectives, we have considered the choice of species very carefully. The real ethical, biological and economic value of the avian relative to other experimental animal models, such as rats and mice, can be best appreciated by considering the lack of need for surrogate mothers or for control of the effects of litter variation or the effects on lactation, as is needed in mammals that give birth to litters and suckle their young. For example, in rodents, one litter irrespective of the number of pups in that litter, is considered as the experimental unit for many statistical comparisons. In addition, adverse pregnancy in mammals may affect the quality of the mother's milk. Therefore, additional control animals, such as surrogate mothers for newborn pups, are needed to understand these effects better. By using the bird as the species of choice to address the project objectives, this markedly reduces the number of animal groups, as there is no need to control for litters or maternal milk. Therefore, this significantly contributes to the 3Rs principle of reduction as enshrined in EU Directive 2010/63.

Where relevant, multiple experimental designs will be used, rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum resource. For most experiments, the study design will adopt methods of analysis previously published extensively by our group, which compares 4 groups: control and experimental groups with and without a treatment or intervention. For example, outcomes from normoxic (normal air) or hypoxic (lower than normal oxygenation) incubations with and without treatment with an antioxidant. Control groups treated and untreated are necessary, as the treatment may affect normal and complicated incubations differentially.

Sex differences are an important consideration in the risk of developing cardiovascular disease. Therefore, assuming a 1:1 ratio of males to females, the number of animals required per outcome variable will be doubled to be able to address sex differences. In such cases, statistical analysis able to compare three factors comparing treatment, intervention and sex will be adopted (e.g. a Generalised Mixed Linear Model; SPSS).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will optimise the number of animals used in this project from 1) pilot data and 2) by using multiple data obtained from the same animal. For instance, we will obtain data from the living organism as well as from tissues isolated from them after death. In addition, we routinely share tissues generated from projects for collaborative studies by other investigators. About 20% of our publication output is derived from such collaborative studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The work will be done in the chicken, which has several advantages. First, it permits isolation of the effect of adverse developmental conditions directly on the fetal heart and circulation independent of effects on the mother and the placenta. This helps better identify potential direct mechanisms and interventions on the developing individual. Secondly, most of the work will be done in the chicken embryo pre-hatching rather than in the post-hatching animal. Thirdly, the work is of greater relevance to humans than using rodents because the development of the heart and circulation is much more similar in humans and chickens, compared with humans and rodents. Fourthly, we can apply interventions in greater numbers of chicken embryos at any one time within one incubator, allowing for contemporaneous comparison and thereby minimising confounding effects of increased variability (e.g. experiments affected by seasonality, effects that sometimes cannot be avoided in longer living species).

A much larger component of the work can be achieved by investigating isolated organs and tissues. For example, after death, the function of the isolated heart and vessels can still be investigated, as well as experiments at the level of the cell and molecule. Comparatively smaller components of the work will involve studying whole living animals under terminal anaesthesia, or conscious animals which have been surgically prepared under general anaesthesia. In some cases, it is necessary to study conscious animals, as anaesthesia can impair normal cardiovascular function. Experiments will only be performed following appropriate post-surgical recovery. We will keep suffering to the minimum by using procedures with the least possible severity, and by subsequent monitoring with veterinary advice.

Why can’t you use animals that are less sentient?

The development of the cardiovascular system in less sentient species, such as in worms or flies is very different than in humans. In addition, the regulatory mechanisms of cardiovascular function in such species is not well understood.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

With experience from previous work, we have streamlined and refined surgical procedures that reduce bleeding, shorten anaesthetic exposure and improve port-surgical recovery. For example, we have slightly raised the bird's head during general anaesthesia to prevent inhalation of any crop reflux. We have also used a different surgical approach to refine procedures. For example, in some preparations, we will need to catheterise the femoral artery of the adult bird. We now do so by isolating the vessel on the outside of the leg muscles rather than through an inguinal approach. From past experience, we have found that this refined surgical approach markedly reduces the disruption of circulation, reduces bleeding and improves the post-surgical recovery of the bird.

In some birds, under recoverable anaesthesia and using strict aseptic conditions, catheters will be placed in one leg and a flow probe in the other. Following 5 days of postoperative recovery, experiments lasting 7-10 days will be performed in these chronically instrumented birds. Approximately 80% can stand following surgery, 15% are reluctant to stand but can stand and weight bear when prompted and 5% are unable to stand. A score sheet has been developed to assess mobility and behaviour to guide decisions, and it will continue to be used and improved as appropriate. The score sheet will be trialled for 6 months and it will be refined thereafter with the input from the Named Veterinary Surgeon.

Bedding in the animal holding areas has also been improved. Some forms of past bedding used in the facility created a lot of dust in the animal's environment. The new bedding material generated much less dust.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure experiments are conducted in the most refined way, we follow the NC3Rs' ARRIVE guidelines, the LASA guidelines and the PREPARE guidelines.

For example, we will refer to the latest edition of the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2017, at the time of preparing this project licence application.

The Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines issued by NORECOPA (https://norecopa.no/prepare) covers all stages of quality assurance, from the management of an animal facility or population to the individual procedures which form part ofa study.

We will refer to specific guidance or position papers from the Laboratory Animal Science Association, (LASA) https://www.lasa.co.uk/current_publications/. For example: Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T (2018) PREPARE: guidelines for planning animal research and testing. Lab Animal 52(2): 135-141. doi: 10.1177/0023677217724823.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We visit the NC3Rs website frequently and have subscribed to their newsletters.

Project licence holders are ultimately responsible for implementing the 3Rs within their work. Therefore, the project licence holder will have regular discussions with the Named Persons and animal technicians to review current approaches and whether there are any new 3Rs opportunities.

We will access our local NC3Rs Regional Programme Manager. We will maintain contact to obtain an informal route to 3Rs advice, developments, and best practice.

We will use of other resources, such as Norecopa https://norecopa.no/databases-guidelines. Regular consideration and reflection of the latest practical guidance from Laboratory Animal Science Association (LASA) will provide additional sources of new recommendations and advances in animal techniques.

179. Maintenance of gerbils with filariasis

Project duration

1 years 0 months

Project purpose

- Basic research

Key words

parasitology

Animal types	Life stages
Gerbils	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To maintain infected gerbils with the human filarial worm parasite *Brugia malayi*

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 continuously maintained a specific isolate of a human filarial worm parasite, *Brugia malayi,* in our laboratory by routine passage between gerbils and mosquitoes since 2012. This lab isolate has been used in a variety of basic and translational research including drug discovery and development for human filariasis. The use of this specific licence will be to continue the propogation of our filarial worm parasite life cycle

What outputs do you think you will see at the end of this project?

The only benefit will be the continued resource of our specific isolate of *B. malayi* which we intend to utilise in the near future for a variety of basic and translational research.

Who or what will benefit from these outputs, and how?

Our laboratory will benefit from continued maintenance of this specific strain of *B. malayi,* in order to carry out future basic and translational research studies

How will you look to maximise the outputs of this work?

This licence will allow us to prolong the life cycle of Brugia malayi so that we may apply for a more extensive licence to cover our next five year research programmes in new anti-filarial drugs and diagnostics

Species and numbers of animals expected to be used

- Gerbils: 10

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult gerbils are the lowest outbred vertebrate susceptible to *B. malayi.* Male gerbils are more susceptible to infection. Male gerbils tolerate infections in the peritoneum without overt pathology. Length of infection/age is limited to 18/24 months to mitigate against decline in welfare due to ageing.

Typically, what will be done to an animal used in your project?

The gerbils have been infected with *B. malayi* on another project licence. The gerbils will be transferred to this licence and maintained. The gerbils will either be humanely destroyed for isolation of parasites or transferred onto a subsequent licence for continued use.

What are the expected impacts and/or adverse effects for the animals during your project?

No expected overt clinical signs or adverse effects due to infection with Brugia malayi via the peritoneal route. Gerbils may decline in welfare due to age, reflective of the long-term maintenance. This may include decline in mobility, weight loss and potential risk of skin bacterial infection. We will monitor any decline in welfare routinely, take remedial actions such as wet food, topical antibiotics and other treatments where appropriate and not allow any animal to suffer more than a mild severity before humanely culling.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

mild, 10 gerbils (100% of procedures)

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?

Brugia malayi has a complex life cycle and requires a mammalian and insect host to develop and reproduce. With NC3R funding we have attempted to culture infectious larvae derived from mosquitoes to grow outside of the body. Our attempts using co-cultures with various mammalian cells has failed to support the growth of these parasites to a reproductive adult stage.

Which non-animal alternatives did you consider for use in this project?

advanced 2D, 3D and organoid *in vitro* cultures to propogate *B. malayi* from infectious stage to mature reproductively active adult worms.

Why were they not suitable?

We have in the past trialled several methods to grow the mammalian life cycle stages of *B. malayi* in complex co-culture systems. These attempts have proven unsuccessful. Whilst we have developed methods to prolong adult *B. malayi* worm survival *ex vivo,* this still requires an animal host to provide a source of parasites. It might be possible in the future, with advancements in *in vitro* organoid models of the lymphatic system (the natural parasitic niche of this parasite), to establish an environment to propogate reproductively active adult *B. malayi.* However, such studies have not been placed in the public domain and we are not currently funded to undertake such expensive and high-risk experimentation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 require maintenance of 10 gerbils in total over a maximum period of 12 months

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

NA

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

NA

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Gerbils are the lowest outbred vertebrate susceptible to Brugia filarial worms. Gerbils tolerate these infections within the peritoneum without signs of disease or ill health. Length of infection/age is limited to 18/24 months to avoid decline in welfare due to ageing.

Why can’t you use animals that are less sentient?

The long life cycle of and requirements to maintain Brugia parasites long term precludes less sentient alternatives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimize development of any welfare issues due to ageing, gerbils will be maintained for a maximum period of +18 months post infection / +24 months of age (whichever is sooner). Through ten years experience, not exceeding these time frames safeguards against the majority of age-related loss of welfare issues which arise. When loss of welfare does arise we will not allow an animal to exceed a mild level of suffering before humanely culling, judged by appearance, behaviour any specific clinical symptoms and/or weight loss.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow best practice guidance for handling, housing and husbandry of gerbils: https://nc3rs.org.uk/3rs-resources/housing-and-husbandry-gerbil

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?
I am an NC3R grantee, an NC3R panel member and chair the institutional AWERB. Through these activities I and receive regular communications from stakeholders such as LASA, NC3Rs and RSCPA. Our AWERB members are proactive in attending off-site seminars on aspects of 3Rs and report back to practicioners. Similarly I receive information and advice from the secondary establishment biological services unit on best practice.

180. Development and regulation of Immunity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immune cells, Therapy, Inflammation, Autoimmunity, Infectious diseases

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We are trying to understand the fundamental processes by which the body's immune system normally develops the ability to fight infections but avoids causing the sort of "friendly fire" that sometimes leads to diseases like rheumatoid arthritis and insulin-dependent diabetes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We need to understand how the immune system responds to infections, why it fails in some patients and how we might modify in a range of diseases. One outcome of our research will be an understanding of how to improve the treatment for cancer by triggering a mild form of friendly fire to tumours and new ways to turn off immunity in diseases like rheumatoid arthritis. We expect to be able help families with rare forms of immune deficiency and our study of these diseases may ultimately inform the design of vaccines.

What outputs do you think you will see at the end of this project?

We will publish information about how the immune system works to fight infection and prevent autoimmune diseases, and our work will help to develop new treatments for human disease. The immune system is directly involved or implicated in multiple forms of human disease, ranging from cancer treatment, to heart disease, schizophrenia and dementia - so our fundamental research will have broad benefits.

Our approaches are designed to gain as much information as possible from the mice, the vast majority of which are used for breeding, so we can select the most informative experiments. These approaches are published and adopted elsewhere.

Who or what will benefit from these outputs, and how?

The beneficiaries of our work in the short term will include the scientific and commercial sectors generating new treatments for human disease; and in the long term the beneficiaries will include patients. Our work is published in scientific journals and at conferences, where it will be read or otherwise communicated and contribute to new approaches for treating disease. Our work contributes to the develoment of new drugs that can be used in the treatment of disease and to new approaches to diagnosis, which may be transferred to human samples generated in the NHS. We also communicate our work through public engagement.

How will you look to maximise the outputs of this work?

We will maximise the outputs by disseminating knowledge through publication, collaboration, lectures and public-speaking. We will continue to publish in top scientific journals and attend conferences where we can explain our findings and exchange ideas withouther scientists. Our collaborations are widespread in the UK and overseas, and our work is also widely read. We will continue a variety of forms of public engagement, including webinars and short films.

Species and numbers of animals expected to be used

- Mice: 50,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used in these experiments will be mice, which are the most appropriate fully developed model of the human immune system - accurate in all important respects but the least sentient. Some of these animals will be genetically modified strains, carrying spontaneous or induced, targeted mutations or transgenes. We will be using mainly adults but occasionally neonates or foetuses, because we also want to understand how the immune system develops in early life.

Typically, what will be done to an animal used in your project?

Some mice will be immunised and rarely mice may be challenged with self-limiting viral infections, such as influenza, to check their immune response. Some mice will also be irradiated to destroy the immune system, followed by rescue with donor cells. Some will be used to model inflammatory disease - such as kidney inflammation - and bone marrow transplantation; and in these models we may test new drugs to ameliorate disease.

The majority of mice used in these projects will kept for breeding, where they do not suffer harm. The other procedures are time-limited with most animals used within 6-18 weeks of full adulthood. Mice will be closely monitored during this period/

Mice used in the regulated proedures are subject to vaccination We expect to use approximately 50,000 mice in 5 years.

What are the expected impacts and/or adverse effects for the animals during your project?

Most experiments (>95%) will not cause any adverse effects. Other experiments may result in mild or occasionally moderate harmful side effects, such as weight loss or transient changes in behaviour due to the immune response brought about by injection of the animals with vaccines or other substances affecting the immune function, vaccination, irradiation, or autoinflammatory processes following infection of autoimmune reactions.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

60% subthreshold

15% mild

25% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are particularly interested in how the macrophages, dendritic, T and B cells that orchestrate the immune response attack infectious particles and how these cells are regulated. Discovering how this happens is a challenge because most of the immune response occurs in inaccessible parts of the body like the spleen, so many of the key advances in this field have been made in mice. The mouse and human immune systems are very similar and share conserved regulatory pathways; and the mouse genome is very

similar to the human, meaning that it is possible to translate findings in genetics between both species.

Which non-animal alternatives did you consider for use in this project?

As far as possible we use model systems using immune cells in vitro to model the effects we want to investigate.

Some of the work leading up to this project was based on the use of in vitro non-animal alternatives, including cell lines; however these lines have been grown without selection for many generations and have accummulated mutations, which make them unreliable. Moreover, these model systems cannot replicate the complexity involved in the immune response, which is seen in animals.

Many of the reagents used in the study - for example, antibodies - are generated in animals but maintained in cell lines; and we use similar model systems to develop our approaches before applying them in animal experiments.

Why were they not suitable?

The normal development of immune cells, which depend on the local environment, and the complex interactions of different cells in different organs during an immune response can only be studied in vivo. In our experiments we are exploring disease processes that occur in the setting of animals and the development of immune cells in the setting of particular tissue environments, such as the bone marrow and spleen. Our experiments are also focused on the effect of genetics on disease, which occurs by natural inheritance. It is not possible to replicated these processes 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?

This is based on our experience and, since the majority of the animals are used in breeding, our plans for generating strains of mice with particular characteristics that can be used to answer specific questions in our experiments. In other experiments, we make calculations that are based on expected differences to work out the numbers of mice required to get a meaningful result - for example, the number of mice to be given and not given a particular intervention.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the smallest possible group sizes that are still informative for a thorough statistical analysis and logical progression to the next step. Advice is sought, and we use various tools and experts in statistics to support these aspects of the programme - for example the NC3R's Experimental Design Assistant. We are very careful to reduce variation by housing the mice together and ensuring that they are genetically identical. Several new

approaches reduce the need for as much breeding, including the use of small number of mice that are first irradiated and then have different bone marrow transplants, so we can compare the immune system that develops from the bone marrow in multiple animals without further breeding; and the maintenance of strains combining multiple genetic differences where these are not harmful. We conduct our experiments in compliance with the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We optimise our colony management to keep breeding cages to a minimum and use a single database to assist us in tracking and managing the breeding strategy. We use ANOVA and other statistical tools to maximise our ability to detect difference in groups with uneven numbers. We use small pilot studies to ensure that projects are feasible and to reinforce our ability to detect difference. We share tissues to make the most of mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of experiments require us to breed and then compare tissues from mice that are normal (wild-type) or genetically targeted to recapitulate human diseases, or to establish the role of proteins and biochemical pathways in immune function. The disease models recapitulate human autoimmune disease like systemic lupus erythematosis, graft-versus-host disease, or kidney disease due to inflammation; or they model human immune deficiency, where this is due to mutations affecting the development of immune cells. These enable us to investigate the underlying mechanisms and possible treatments of the same conditions in humans. The pathways may be perturbed in human disease or be potential targets for therapy. The breeding protocols cause minimal or no distress. Other experiments are designed to deliver statistically significant results in the least harmful way by making power calculations and focusing only measurable differences in well matched mice and controls. We use bone marrow transplantation, which is well tolerated, as a way of generating several well-matched mice with identical immune systems, without the need for excessive breeding, and we use models for infection, inflammation and autoimmunity when these have been very well characterised. In the bone marrow chimeras, we frequently mixed wild-type and mutant bone marrow, so we can compare the development of cells side-by-side in single mice. In our experience this is an extremely sensitive way to pick up differences and the refinement results in a reduction in total mice. We continually seek ways to improve these assays and reduce the level of suffering to the animals.

Why can’t you use animals that are less sentient?
Mice are the least sentient animals available with multiple reagents for conducting experiments and exploring hypotheses, including transgenic and knockout strains, for studying a complex adaptive immune system that is similar to humans. Non-mammals do

not recapitulate the human immune system as well as mice, which only diverged from us 80 million years ago in evolution.We study the development of the immune system in neonatal and adult mice and the response to antigens (vaccination) and self-tolerance in adult mice, which requires us to observe live individual animals over several days or weeks.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will review our procedures on a regular basis in our weekly lab meetings and share information with other groups and through our NC3R committee to establish best practice and continue to minimise the harm to the animals. We refine our work by reading the literature, listening to presentations in the field, and through this process of sharing and our weekly lab meetings. Examples include taking the data from one experiment and using it to revise the number of animals and the schedules of interventions and monitoring in the subsequent tests.

We will review the cost: benefit of all our experiments throughout the lifetime of the licence.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow published protocols which are assessed by our AWERBs in consultation with our veterinary surgeons. Some of the other resources we use include www.nc3rs.org.uk; https://norecopa.no; https://www.lasa.co.uk.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We follow and contribute to our local NC3R committee and attend committees for local users, where we share data and methods. We follow activity at www.nc3rs.org.uk and https://science.rspca.org.uk. We will follow best practice as it evolves. We monitor our animals closely. We will exchange information on animal welfare by engaging with NACWOs, Veterinary Surgeons and other investigators in our institute and outside it. We seek advice from our colleagues. We keep careful records so we can refine our protocols to be more efficient over time. We keep our training up to date and adopt new methods when they will improve animal care.

181. Mechanisms and rescue of absence seizures and their psychiatric comorbidities

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

non-convulsive seizures, cortico-thalamic networks, cortico-hippocampal networks, pharmacological therapy, psychiatric comorbidities

Animal types	Life stages
Mice	neonate, juvenile, adult, embryo, pregnant
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's the aim of this project?

To characterize the mechanisms of generation, propagation and termination of absence seizures and their neuropsychiatric comorbidities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Absence seizures are genetic generalized seizures that consist of sudden, relatively brief lapses of consciousness, lack of voluntary movements, a blank stare and 2.5-4Hz spike/polyspike-wave discharges in the electroencephalogram. Absence seizures are the only clinical symptom in childhood absence epilepsy, highly prevalent in paediatric/juvenile populations and often present with other seizure types in many age-dependent and age-independent epilepsies with greater severity and less positive clinical outcome.

Absence seizures are still considered relatively benign because of their non-convulsive nature and relatively high remittance rate in early adulthood. However, recent studies in large childhood absence epilepsy cohorts have shown that 30% of these children are pharmaco-resistant (a rate similar to that of more severe seizures), leading to polytherapy and consequent increase in drug adverse effects.
Moreover, 60% of children with absence seizures suffer from psychiatric comorbidities, including deficits in memory and attention that can persist after full pharmacological control of the seizures and be aggravated by some anti-absence drug. Notably, it is not known whether these comorbidities are causally linked to absence seizures or develop independently from the epileptic phenotype.

Thus, there is a compelling clinical need to discover novel therapeutic targets that may control both absence seizures and their comorbidities. However, though advances have been made in recent years to understand the pathophysiological mechanisms of these non-convulsive seizures, we still do not have a full picture of the mechanisms that control their generation and termination. Moreover, very few studies have addressed the origin and mechanisms of the comorbid psychiatric impairments that are present in children, teenagers and adults with absence seizures.

What outputs do you think you will see at the end of this project?

The primary output of this project will be the characterization of the mechanisms of generation and termination of absence seizures and their psychiatric comorbidities. Moreover, this project will identify targets that control both absence seizures and their comorbidities, leading to the development of single pharmacological entities that could rescue both the primary and secondary phenotypes of this neurological disease.

Who or what will benefit from these outputs, and how?

We expect early-stage (Phase 1) clinical studies on the use of endogenous substances against absence seizures and their comorbidities to be initiated towards the end of this project, whereas the synthesis and testing of novel exogenous substances may require longer efforts (5-10 years). Children, teenagers and adults with absence seizures will be the main cohorts benefiting from this project, including individuals with childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy, epilepsy with myoclonic absences, Dravet syndrome and Lennox-Gastaut syndrome.

How will you look to maximise the outputs of this work?

The primary output of this project will be the publication of original papers in high impact journals describing the technical and scientific discoveries that we will make. Any potential improvements in the 3Rs will also be similarly publicized in relevant journals. Moreover, both technical, scientific and 3Rs output will also be disseminated at international neuroscience and epilepsy conferences.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We will use the best available genetic mouse and rat models of absence seizures, that have strong face-, construct- and content-validity. Some work will be done in juvenile animals but other experiments will be performed in animals older than 3 months of age when absence seizures are fully developed.

Typically, what will be done to an animal used in your project?

Animals (~40%) will be terminally anaesthetized for *in vitro* histological, immunological and biochemical analyses of their brain.

Animals (~30%) will be implanted with EEG electrodes (under general anaesthesia) to test the effect on absence seizures of endogenous substances, anti-epileptic medications or drugs that modulate neuronal ion channels, transmitter receptors and transporters.

Animal (~30%) will undergo a series of behavioural tests to assess their memory and learning deficits and the effect on these impairments of endogenous substances, anti-epileptic medications and drugs that modulate neuronal ion channels, transmitter receptors and transporters.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect major long-lasting adverse effects resulting from the proposed procedures, though some temporary post-surgery discomfort may occur in some animals. Injection of a substance may also cause minor temporary discomfort. All substances to be used in these experiments will have been already tested in normal, i.e. non-epileptic, animals to confirm and establish their site and mechanism of action.

Fifty percent of the genetically altered mice may experience moderate harms due to an ataxia phenotype which involves loss of balance and coordination.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

30% of animals will experience subthreshold harms only - breeding without harmful phenotypes and genotyping by an unregulated method

20% of animals will experience non-recovery harms - due to anaesthesia induction

20% of animals will experience harms of a mild maximal severity due to surgery and/or drug injections. 25% of animals will experience harms of a moderate maximal severity due to an ataxia phenotype
5% of animals will experience harms of a moderate maximal severity due to surgery and/or drug injection.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Existing computer-programs that simulate the rhythmic electrical activity underlying absence seizures are highly inadequate in describing absence seizure firing patterns and associated membrane potential changes, because they lack data on single-neuron biophysics and network-wide firing interactions, i.e. the very same knowledge that our project aims to collect. Absence seizures cannot be reproduced in cultures of immortalized human cell lines or in primary animal neuronal cultures.

Though the technology for differentiating human fibroblasts and stem cells into excitatory and inhibitory (mainly cortical and mesencephalic) neurons are at present being developed, the complexity of absence seizure generation would require the differentiation and culturing, and successful interaction, of many different neuronal and astrocytic cell types from various brain regions: this, unfortunately, is still not available.

Which non-animal alternatives did you consider for use in this project?

In silico (i.e. computer-based) experiments, work in immortalized human cell lines and stem-cell- differentiated cell cultures. As indicated in the previous answer, these are all inappropriate for this project.

Why were they not suitable?

They are unable to reproduce absence seizures and their psychiatric comorbidities.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals has been mainly derived from our 40 years' experience with similar types of animal experiments that were supported by previous Project Licences. Our current funds support three main research lines within the general "umbrella" of the "pathophysiology of absence seizures". Successful completion of one of these research lines earlier than anticipated may result in a reduction of this estimate.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3R's Experimental Design Assistant and the ARRIVE guidelines have been used, together with our long-standing experience in this field, to reduce the expected number of animals.

Notably, the epilepsy research-selective guidelines (whose publication I contributed to) were also used in the planning of the experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible, the biochemical, histological and immunocytochemical analysis will be carried out on brain tissue harvested from animals that have completed other tests.

Our 40 years experience of using rat and mouse models of absence seizures has led to a very efficient breeding of these colonies with minimal 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The vast majority of the experiments will be carried out in the best available mouse and rat models of absence seizures, i.e the stargazer STG mice and the inbred Genetic Absence Epilepsy Rats from Strasbourg (GAERS), both of which have been shown to have strong face-, construct- and content- validity, i.e. to be valid models of this neurological disorder.'

The GAERS rats are superior to other available rat models since they have their own inbred control, non-epileptic strain (generally referred to as the NEC rat strain) to be used for comparative results. Moreover, the discovery of the cortical regions where absence seizures originate (before becoming generalized) was made in GAERS rats before being observed in children with absence seizures.

STG mice, as all other single-mutation mouse models of absence seizures, have a mild ataxia (problems with balance and movement coordination) phenotype.

The most selective drugs, the best transgenic species and strains are always selected even they are often expensive to purchase and maintain, respectively.

In vivo experiments are never carried out until we have solid *in vitro* data, thus ensuring optimal use of the *in vivo* preparations.

Why can't you use animals that are less sentient?

Absence seizures are developmentally regulated (both in humans and in animal models), i.e. so that they can only be appropriately studied in mice and rats that are older than 3 months.

Terminally anaesthetized animals cannot be used since both in humans and in experimental animals absence seizures are fully blocked by by general anaesthesia. New-born and juvenile animals will be used when assessing the developmental profile of abnormalities in brain receptors, ion channels and transporters.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Continuous monitoring and advice from animal care staff and NVS will ensure optimal housing and welfare conditions, in particular for the chronically implanted animals.

A high standard of environmental enrichment are consistently provided before and after any procedure, except in special cases, i.e. in chronically implanted animals.

Pre- and post-operative pharmacological control of pain and inflammation is consistently implemented.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I was instrumental in setting up a working group on the best practices for animal experiments in epilepsy. The working group included UK researchers in epilepsy, NC3Rs representatives, experienced NVSs from different national institutions, and world experts in epilepsy research from Europe and the USA. Moreover, Home Office inspectors attended, and provided input to, some of this group's meetings. I will follow the guidance that this committee published at the end of their work since it still represents the newest guidelines in epilepsy research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I received continuous updates from the NC3Rs newsletter and via the local Regional Programme Manager. Any advance in refinement techniques and methods will be carefully considered for implementation in consultation with local animal welfare personnel, provided their implementation would still allow comparison of results with previously obtained data.

182. Animal models of fibrotic 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

Fibrosis, Liver, Kidney, Experimental therapeutics

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to provide a demand-led service for the delivery of new treatments for patients with fibrosis. This project will test the efficacy of antifibrotic drugs in validated rodent models, to refine and optimise the use of therapeutics for the treatment of fibrosis in humans. The data generated from our studies will impact the drug development process, support new drug applications, and allow drugs to progress to the 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?

Fibrosis occurs in response to injury when the tissue repair response becomes uncontrolled, leading to thickening and scarring of the tissue, and organ dysfunction over time. Fibrosis can affect any organ, including, skin, lung, liver and kidney. It is a major cause of death worldwide and it is often seen in chronic inflammatory disorders, such as metabolic syndrome and cardiovascular disease.

Unfortunately, to date, only two drugs are available for the treatment of lung fibrosis, and no treatment has yet been approved for liver or kidney fibrosis.

Despite substantial progress in our understanding of the nature of fibrosis, a gap remains between the identification of antifibrotic targets and conversion of this knowledge into effective treatments in humans. Therefore, our preclinical data will pave the way to predict successful clinical trials and help pharmaceutical companies decide which drug(s) to develop.

What outputs do you think you will see at the end of this project?

This project will provide data from various pre-clinical models to biotech and pharmaceutical industries, to identify those compounds most likely to have the greatest clinical potential and highlight those with limited efficacy results.

The drug development in the field of fibrosis remains limited, hence offering this work as a service, represents an extraordinary opportunity to yield new discoveries and drive a new era of precision medicine in the treatment of chronic fibrotic diseases. In the long run, the project's potential outputs are human health improvement, thus reducing the burden on healthcare.

Who or what will benefit from these outputs, and how?

The principal benefit of this project is to identify promising next generation treatments for fibrotic disorders benefiting patients with multiple fibrotic conditions. The short-term benefit of the data generated from studies is to facilitate the drug development process. In medium-term the data generated is used to support new drug applications, and the long-term is to enable drug treatments to progress to the clinical trials.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, we will share the results of the project through publications and preprints when appropriate and possible, in line with the establishment policy for the use of animals in research. We will also look into building new relationships, both in the field of fibrosis and beyond, as the knowledge generated through this project can be easily applied to other diseases.

We have an extensive in-house experience of drug testing *in vivo*. We can provide to our commercial sponsors our in-depth knowledge of fibrosis and *ex vivo* tissue analysis to identify biochemical markers that better correlate with disease outcome. This will maximise the quality of the data and provide a broad census of cells involved in fibrosis. Crucially, this data will support our sponsors with increased diagnostic accuracy and prognosis in clinical trials.

Species and numbers of animals expected to be used

- Mice: 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The programme requires that models are used, which closely mirror the human biological system and can capture the potential therapeutic effects on fibrosis and inflammation. Adult mice are suitable for these studies as the work cannot be conducted in lower vertebrates, invertebrates, or cell lines due to the poor resemblance of these options to the clinical setting.

Typically, what will be done to an animal used in your project?

The bulk of the animals in this protocol will undergo at least one or two (100%), or up to four (50%) of the following procedures listed below:

Animals will be obtained from established providers and acclimatised for at least 1 week before studies are initiated.

Animals will be treated with agents that cause liver and kidney fibrosis. For all models, animals will be monitored daily and weighed at least 2-3 times a week, unless more frequent weighing is warranted.

Test Item (or the same solvent without any drug, if using controls) will be administered via a known route, and at a volume, concentration and schedule that is known to be well tolerated.

Blood samples may be collected periodically via a tail vein bleed in conscious animals. We will always follow the NC3Rs guidelines/limits for peripheral blood sampling. We may collect urine samples so that we can measure changes in kidney function associated with fibrosis (e.g., presence of different chemicals in the urine and urine volume). We will employ the most refined method of collection of urine, to include urine droplet, but where collection over 24 hours is required, mice may need to be kept in 'metabolic' cages.

In some instances, tissues from animals may be collected for studies in the laboratory (*ex vivo* studies) under terminal anaesthesia.

Fibrosis of the liver may be induced by injection of carbon tetrachloride into the abdominal cavity. This chemical is metabolised in the liver and converted to a highly reactive form, that causes liver damage, resulting in inflammation and fibrosis. This model of advanced chronic liver disease is one of the most widely performed and can also be used to study fibrosis regression as the damage caused by the chemical is partially reversed when exposure stops. Mice may be given a high fat diet prior to chemical exposure since liver fibrosis often overlaps with obesity. This model is useful to study toxic-mediated liver fibrosis and mimics acute liver injury in humans.

Non-alcoholic steatohepatitis (NASH), is a condition where fat accumulation causes liver inflammation and scarring (fibrosis). In order to mimic this disease, mice may be fed with a nutrient-deficient diet (choline- and L-amino-acid deficient), high-fat and cholesterol diet for a period of 12-16 weeks. Control mice will receive a matching diet. This model is of great significance to understand how lifestyle and nutrition play a role in the development of fibrosis.

Renal fibrosis i.e., fibrosis of the kidney, may be induced by feeding animals a diet rich in adenine for 2-4 weeks. This model allows the study of mechanisms involved in renal

dysfunction, similar to humans with chronic kidney disease. This model is relevant to understand renal fibrosis since inflammation and fibrosis markers can be detected during the progression or resolution of the disease when the diet is switched back to normal.

What are the expected impacts and/or adverse effects for the animals during your project?

Like humans, animals can sustain fibrotic injury for a long period of time with no apparent symptoms. In the rare scenario that an animal shows signs of organ failure we will ensure that the animal does not exceed the severity limits set out in this project. Previous experience has shown that these procedures typically induce mild to moderate adverse effects:

Animals will experience stress due to restraint and transient discomfort from needle insertion and/or anaesthetic injection or inhalation of gaseous anaesthetics (100 % incidence).

Repeated intravenous injection can result in irreversible damage to the vein and very occasionally, a haematoma/bruising may develop.

Intraperitoneal injection (through the abdominal wall) is likely to be painful if the needle injures an abdominal organ or if the substance being injected is an irritant.

Oral administration (gavage) is associated with minor discomfort. Very occasionally damage to the oesophagus may occur or substances may enter the lungs resulting in difficulty in breathing.

Slow-release pellets and osmotic minipumps are small devices implanted under the skin of the mouse and cause minimal effects. Animals may experience a lowered body temperature during anaesthesia required to implant these devices. Animals may experience pain on recovery from anaesthesia.

Animals will experience stress due to restraint and a transient discomfort from blood collection (100% incidence). Animals may become overheated or dehydrated from spending too long in the hotbox.

With all surgical procedures there is some risk of development of infections or wound complications (<1%).

Animals fed with high-fat diet will develop obesity (up to 100%) and greasy coat (up to 100%), which may lead to over-grooming (~25%) and as a result possible skin inflammation/ulceration and infection (expected incidence of <5%).

For a diabetic animal, if diabetes is ongoing, diabetic kidney disease, and high blood sugar will occur (up to 100%). For an obese animal without diabetes, weight gain can result in hypertension and respiratory depression.

Animals with liver or kidney damage may lose 10-15% of body weight (up to 70%).

Mice with kidney disease will show proteinuria (high quantities of protein in the urine) (100%).

Mice with liver disease could show abdominal swelling, although this is rare and extremely variable.

Animals may show cardiovascular or gastrointestinal system dysfunction (<10%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Given the controls in place, the expected severity for animals under all protocols under the licence is moderate and may be experienced for up to 100% of animals used.

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?

Fibrosis is a highly complex process. Producing a non-animal alternative in the lab, which replicates all aspects of fibrosis, is currently impossible. Due to the complexity and ever-changing physiological environment during fibrosis, the use of mice is necessary as the tissue architecture plays an important role in influencing this process. Currently, it is inconceivable that we will be able to generate computer models that will allow us to study the cell-cell interaction in an ever-changing three-dimensional structure that is required for this study.

The animal models proposed for this study, have been carefully selected to allow effective drug development and reduce animal pain. Although these models are specific for kidney, and liver, other organs such as pancreas, gut, heart, bone marrow, and skin can be analysed in one model. Therefore, this approach can address the current scientific need and minimize the number of animals required in each experiment.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives such as drug testing on liver organoids and *in vitro* assays using fibroblasts, hepatocytes or cell co-cultures are being successfully established in the lab. We have invested heavily in *in vitro* model systems over the last years, purchasing new analysis platforms for the laboratory and fostering collaboration with other laboratories.

This technology has been considered for initial drug screening, which may help to partially replace the number of animals used. We have focused on developing 3D cultures, derived from tissue samples obtained from a range of species, including mouse and human. These cultures allow us to look at how drugs directly affect the digestive system and help us identify potential novel treatments.

Why were they not suitable?

Liver and kidney fibrosis alternatives from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) will be considered. However, cells studied *in vitro* do not offer a true reflection of inflammation and fibrosis that animals can provide. Furthermore, common variations in culture conditions can have large effects on cells or organoids and have to be taken into account when designing experiments and comparing results. Therefore, the use of animal models is vital to facilitate disease study and subsequent drug screening.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Data from previously published studies has been used to estimate the total number of animals in each protocol. To obtain the desired effect, the number of animals estimated has been calculated based on the accumulation of type I collagen. Where possible we will make use of organoids or archived tissue to reduce animal use.

Therapeutic compounds studied under this licence will have demonstrated *in vitro* efficacy and will have some supporting toxicological data. Only those compounds showing reasonable activity and specificity for their intended target will be considered for animal testing.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have greater than 5 years of experience using mice as a model system to study fibrosis. I have used my experience together with statistical advice and online tools such as the NC3R’s to minimise the number of animals used for testing our hypothesis. Furthermore, prior to the experimental design, I used the 3R's site (www.3rs-reduction.co.uk), the PREPARE guidelines (https://norecopa.no/prepare) and had input from statistical experts to minimise the animal used while ensuring high reproducibility.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Academic experts and independent companies are consulted to ensure the rigour of our experimental design and analysis. We are promoting multi-user studies to improve the efficiency of animal usage. We will stay informed about advances in the 3Rs (reduction, replacement and refinement), and implement these advances effectively during the project.

We will also have a fibrosis database to ensure that every organ collected will be recorded together with the relevant information (e.g., experiments conducted, age, sex) to ensure that it can be used when needed. In addition, the tissue collected can be used for pilot experiments. Furthermore, we will encourage clients to also share and publish negative

results to avoid unnecessary duplication of studies. Together, this will reduce the overall usage of mice in our experimental procedures while sharing the information with everyone in the university when possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models of fibrosis are well-characterised and demonstrate broad similarities with human disease, regarding symptoms and fibrotic changes. The models included in this project have been selected based on the disease origin. Furthermore, the least damaging models with appropriate translation into human disease are being selected.

There are other models available such as the rodent cafeteria diet (feeding animals a choice of food items to stimulate energy intake) or nutrient-depleted diets (e.g., methionine- choline-deficient diet), but they have not been included in this licence application due to insufficient fibrosis or excessive body weight loss, respectively.

Surgical fibrosis models have not been included due to severe adverse effects and distress. Instead, more refined, and less harmful models have been selected in the interest of maintaining relevance to human pathology.

Why can’t you use animals that are less sentient?

Mouse models are well-characterised and demonstrate broad similarities with human disease, regarding symptoms and fibrotic pathological changes, which non-mammal species do not adequately display.
Animal models have facilitated many discoveries that translated to humans, such as antifibrotic drugs and metabolic regulators of liver health. Examples are the glucagon-like peptide-1 (GLP-1) analogues, the farnesoid X receptor agonists (FXR) and the dual chemokine 2 and 5 receptor antagonist (anti- CCR2/CCR5) that currently are in development for the treatment of liver fibrosis (published studies).

Decades of research have provided substantial standard techniques to evaluate fibrosis in rodents, making mice the most suitable species for the studies proposed for this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animal handing and care will be conducted in a manner that minimise stress whenever possible. Other welfare measures will be:

Gavage: We use specifically designed gavage tubes, and may pre-coat our gavage needles with palatable lubricant, which has been shown to reduce the stress associated with the procedure and reduce gavage time.

Injections: Repeated injections will be not performed on the same site. We will alternate between the right and left side to reduce pain and discomfort. Furthermore, localised administrations and manipulations will reduce the adverse effects that are typically associated with systemic manipulations where multiple organs and cell types are affected at the same time.

Refinement can also be achieved in the type of mice we used. Animal genetic background will be considered to minimise harm. Very sensitive mice strains to the fibrosis model will not be used.

Special care will be given to all animals after a surgical procedure or prior to any animal experimentation where they will be gently handled in order to minimise stress caused by the procedures. This will include increased monitoring after any surgical procedure which includes post-operative care and analgesic if required. In addition, we aim to reduce the duration of all surgical procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure experiments are conducted in the most refined way, we will follow the Animals (Scientific Procedures) Act 1986, the Joint Working Group on Refinement ‘Refining Admin of Subst’ Lab Anim 2001, the PREPARE Guidelines, and the non-technical summaries found in: https://www.gov.uk/guidance/research-and-testing-using-animals. We are keeping up to date with the literature within the field of pre-clinical fibrosis research and are actively looking to implement refinements of our models.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have signed up for newsletters from FRAME, NC3Rs, and www.nlm.nih.gov/toxnet, a cluster of databases on toxicology & hazardous materials, to support our research plan, reduce animal numbers and refine the use of toxic compounds to induce fibrosis. The NORECOPA portal and the PREPARE guidelines will be regularly consulted for design planning.

183. Neural Mechanisms of Autonomic Control

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Autonomic control, Hypothalamus, Torpor, Critical Care, Homeostasis

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, aged, embryo
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify neural mechanisms that regulate autonomic function with a particular focus on torpor, and to develop strategies to modulate these circuits with the aim of improving patient care during critical illness.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the United Kingdom around 200,000 critically ill patients are admitted to an intensive care unit (ICU) annually (www.icnarc.org). Of these patients almost 40% require life support involving mechanical ventilation under anaesthesia. During the current COVID-19 pandemic the UK demand for ICU beds increased 2.5 fold, with 72% of the COVID-19 patients requiring mechanical ventilation.

Admission to the ICU for organ support represents a state in which the demand for oxygen and nutrients is either unmet, due to respiratory or circulatory failure, or else oxygen and nutrients cannot be utilized by cells due to bioenergetic failure. Traditionally, ICU treatment has focused on redressing this imbalance by increasing oxygen and nutrient supply through fluid infusion, blood transfusion, cardiac support with drugs and mechanical support of ventilation. These approaches are far from perfect and frequently result in harm as efforts are made to increase supply to normal or even above normal levels.

An alternative approach, which balances the limitations in supply against the demand by reducing the metabolic requirement of the body could be beneficial in the treatment of many conditions seen in ICU. By reducing metabolic demand, patients may better tolerate critical illness avoiding the need for invasive interventions such as mechanical ventilation. As well as potentially preventing some of the harms from organ support, by reducing the intensity of the care required, this approach would also result in a reduction on the demand for ICU beds and staff.

Our understanding of the autonomic responses to the physiological challenge of critical illness is incomplete. We do not know which responses are beneficial and which are harmful. This lack of knowledge represents a fundamental challenge to optimising the management of life support for patients.

Torpor is a naturally occurring state that is like a short term hibernation (animals become cold, have a very low metabolic rate and appear to sleep), which in mice serves as a protective response to environmental challenge (such as food scarcity). The work conducted under this licence aims to advance understanding of the neural basis of the control of torpor. In so doing, we aim to identify the mechanisms behind beneficial, protective autonomic responses, with the ultimate aim of translating that understanding to develop better strategies for the treatment of critically ill patients. The findings of these studies will be of value to neuroscientists, patients, clinicians, and industry.

What outputs do you think you will see at the end of this project?

This study will advance understanding of:

The neural pathways within the CNS involved in thermoregulation, metabolism, and cardiorespiratory control.

Neuronal mechanisms of torpor control, and the extent to which analogous circuits exist in species for which torpor is not an extant behaviour.

The reciprocal interactions between physiological states such as torpor and circadian rhythms.

and to relay the knowledge gained to scientist and clinicians through publications in the scientific literature and conference presentations. In so doing the work aims to identify protective autonomic nervous system responses that may be manipulated to improve outcomes in clinical situations such as major surgery or critical illness.

Who or what will benefit from these outputs, and how?

The chief beneficiaries of the study outputs will be the academic, clinical, and industrial research communities, who will use the knowledge to drive future research and deploy new therapeutic approaches. Data generated will be published in high impact peer-

reviewed journals (as well as on preprint servers such as BioRxiv) and presented to appropriate audiences at national and international meetings. We will communicate our findings at public and patient engagement events and include patients as partners during the development of our research strategies. We expect in the long term that our outputs will benefit surgical and critically ill patients, and the wider society.

How will you look to maximise the outputs of this work?

We will continue to collaborate with academic research colleagues at both a national and international level to best exploit our research findings – a large part of our work involves international collaborations. We will disseminate our research through traditional peer reviewed publication and scientific meetings but will also publish our research (successful or otherwise) as open access pre- prints and will share the data via appropriate repositories. We will present to the public and at patient engagement events. We will protect any intellectual property so that it can be developed commercially and turned into treatments.

Species and numbers of animals expected to be used

- Mice: 1330
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents have been chosen for these studies because they have the least neurophysiological sensitivity of species within which mammalian autonomic control can be modelled, including the natural display of torpor. Rodents share many of the anatomical and physiological aspect of the human nervous system and consequently have been used extensively in similar studies worldwide. Non- mammalian species (e.g. arthropods and fish) are not appropriate as they either lack the brain structures of interest or, if present, lack the organisation and connectivity with higher centres that we seek to understand. Additionally, they have different neurochemical mediators and distinct receptors, so their pharmacology is often not directly comparable to human.

Both mice and rats will be used for these studies. Mice offer the advantage of naturally displaying torpor and are easily genetically manipulated. This allows the study of specific populations of neurons to define their role in autonomic control. Rats on the other hand offer the advantage of not naturally displaying torpor, but in many other respects are like mice. This allows investigation of the extent to which protective torpor-inducing circuits that exist in mice are present in species for which torpor is not an extant behaviour. Additionally, their larger size and greater cognitive ability offers advantages for some types of experiments.

Typically, what will be done to an animal used in your project?

The majority of animals used in these studies (~75%) will undergo a surgical procedure. In most cases this will involve the injection of a viral vector into a specific region of the brain.

Following surgery, these animals will be returned to a group housed environment for 2 to 4 weeks to enable sufficient expression of new proteins to allow recording or manipulation of neuronal activity. A subset of animals (20%), will have either a fibre optic cable or electrodes implanted into the brain, and/or implantation of a recording device into the abdomen to allow remote recording of core temperature, activity, blood pressure or brain and heart activity (~10%). These surgical steps may be undertaken together or independently, but no animal will undergo more than two surgical procedures in the course of the study. Following surgery, animals may be temporarily singly housed, while the skin heals, before returning to group housing.

Approximately 40% of animals, most of which will previously have received a viral vector injection, will undergo a period of food restriction lasting for up to 5 days during which food intake will be reduced to 70% normal and delivered at a specific time point, usually at the beginning of the active phase at lights off. In mice, this protocol reliably induces a state of torpor, typically by day 3 or 4. In rats, torpor is not induced but the food restriction activates the associated autonomic control pathways. Welfare and body weight will be assessed daily with weight loss limited to 20% from baseline.

Circadian rhythms will be assessed using a running wheel and/or video or remote recordings (~20%). Animals undergoing circadian rhythm analysis will be exposed to a period of total darkness (up to 5 days) in order to assess the impact on their sleep-wake cycle. A small proportion of animals (<10%) will be sleep-deprived for up to 4 hours (e.g. by introducing novel objects to their cage), which will be followed immediately by a period in which recovery sleep is allowed - this will allow the assessment of whether torpor shares some similarities to the neural circuits that regulate sleep. Another group of animals (5%) may be placed in either a warm (up to 30°C) or cool (16°C) environment for up to 5 days, which will tend to increase or decrease the probability of inducing torpor.

Animals may receive intra-abdominal injection of drugs to selectively induce gene expression or to alter neuronal circuit function.

At the conclusion of the study the animals will be killed, and their tissues further assessed ex-vivo using preparations such as working heart brainstem preparation (~30%), isolated heart function and tolerance (~20%); mitochondrial function assays (<10%), and/or histology (~80%). These ex-vivo experiments will be conducted to better define the neural circuits of torpor and also to identify any protective effects on organs.

What are the expected impacts and/or adverse effects for the animals during your project?

All animals undergoing surgery are expected to experience some pain following recovery from general anaesthesia. To mitigate against this, all animals will be given post-operative pain control, which will be maintained until the animals are showing no further signs of pain. All animals are expected to make an uneventful recovery from surgery and to resume normal behaviour within 24 hours.

Food restriction will induce a temporary state of hunger but is not expected to adversely affect the long- term wellbeing of the animals. Weight loss will be monitored daily and restricted to a maximum of 20%. Animals losing >20% of baseline weight will immediately be given ad-lib access to food, and those not regaining weight will be culled at 24 hours. For mice, entry into torpor is a natural and protective behaviour with no known negative impacts. Studies involving sleep deprivation, circadian rhythm assessment and modulation of the ambient temperature are not expected to generate any adverse effects.

Chemo- or optogenetic manipulations of autonomic function are expected to result in reversible changes to core temperature, cardiorespiratory function, and metabolism. These will all be via activation of endogenous circuits and are not expected to adversely affect the long-term wellbeing of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rats: moderate (100%)

Mice: moderate (70%);

Mice Mild (25%)

Mice: non-recovery (5%)

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of the work is to advance understanding of the autonomic control of protective responses (such as torpor) with a focus on identifying those with the potential for translation into a clinical setting. It is not possible to studies these control mechanisms without using living animals as they involve integrated processing by many classes of neurons, located both peripherally and centrally, as well as endocrine factors which only exist in intact living animals.

Which non-animal alternatives did you consider for use in this project?

We have considered computer modelling and recordings from cultured cells such as stem cell derived neurons as alternatives. We also considered the use of human models. None of these provide viable alternatives to the use of animals at this stage, although in the future we would hope to translate our findings to humans and build computer models of the neuronal circuit functions.

Why were they not suitable?

Computer modelling requires biological data to build informative models and make accurate predictions – at present there is no adequate model of autonomic neurophysiological control that has any predictive validity. Cell cultures cannot be used to

make inferences about autonomic control as this requires an intact nervous system integrating information from many sources. Human studies have clear limits in the ability to isolate and control the experimental conditions (through exposure, insertion of recording and stimulating electrodes and the use of genetic and pharmacological tools to probe neuronal circuits) to definitively identify neuronal mechanisms, and at present we do not know the neuronal circuit(s) to target in a human setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have considerable experience in the study of autonomic control mechanisms including the use of chemo- and optogenetic paradigms and behavioural assessments. Specifically, we have been conducting torpor experiments over the past 4 years and the effects on physiological parameters of interest are large (like temperature, heart rate and metabolic function). We have used this information, as well as data from published studies, to estimate the numbers of animals needed (and so group sizes are typically between 6-10 animals).

For experiments using novel measures we will use data from the existing literature where available and conduct pilot experiments (n=3-6) to get an estimate of the necessary number of animals for the definitive study.

For the GA colony estimates these animal numbers have been scaled to take account of whether the strain of animals will be homozygous or heterozygous for the gene of interest (wherever possible we will use homozygous breeding lines). We have experience breeding GA lines for use in this type of experiment, and have calculated the number of breeding pairs required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will make extensive use of within animal controls which halves the number of animals needed for a study and reduces the variance so increasing the power to detect effects. We will use inbred strains of animals to further reduce variance. We will use animals of either sex and will stratify on this variable in our analyses to identify any sex specific differences. We use standardised surgical approaches and follow defined protocols for the adjustment of environment or food delivery. We have made use of the NC3Rs experimental design assistant when planning our investigations and making proposals to funders. Following in-vivo studies and killing, we will then use their organs in ex-vivo preparations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible we will use homozygote lines of GA mice. We will make use of pilot studies for any new experimental protocols. Tissue from animals will be used for ex vivo

experiments such as the working heart brainstem preparation or in vitro experiments such as mitochondrial oxygen consumption assays.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a well-established protocol for torpor induction by food restriction. This protocol results in less than 20% body weight loss across 5 days, indeed once torpor appears, weight loss plateaus as it is an effective energy conserving strategy. For studies of circadian function, we will induce torpor by calorie restriction or by targeted activation of specific central circuits. To assess the impact on circadian rhythms this will then be followed by monitoring in an environment free of external time clues for up to 5 days, necessitating a deviation from normal housing conditions.

Most animals will undergo injection of viral vectors into the central nervous system (CNS). Where appropriate, animals may be implanted with telemetric devices to enable the recording body temperature, activity etc without needing to handle the animals, thereby minimising distress whilst maximising the data yields. A small number of animals will undergo implantation of cannulae, fibre- optic probes or recording electrodes into the CNS or into a blood vessel to allow delivery of drugs, stimulation and/or recording. These experiments will necessitate periods of tethering during recordings.

A minority of animals will undergo indirect calorimetry (up to 24 hours) and/or spirometry (up to 4 hours), necessitating their placement in a plexiglass box.

In all cases the least severe model, consistent with obtaining the necessary data, will be used. For each protocol we have clearly defined humane end points.
These approaches will allow us to study how the brain controls torpor, and how torpor interacts with other autonomic processes, as well as to study whether torpor-like states can be triggered in animals that do not naturally enter torpor.

Why can’t you use animals that are less sentient?

Rodents have been chosen for these studies because they constitute the least sentient species within which mammalian autonomic responses can be modelled. Non-mammalian species (e.g. arthropods and fish) are not appropriate as they either lack many of the brain structures of interest or, if present, lack the organization and connectivity with higher centres that we seek to understand. Additionally, they have different neurochemical mediators, ion channels and distinct receptors so their pharmacology and physiology are often not directly comparable to human. Rodents share many of the anatomical and physiological aspect of the human nervous system and consequently have been used extensively in similar studies in many other laboratories worldwide. We will make use of ex vivo experiments for some measurements. By using reduced preparations e.g. the working

heart brainstem preparation to address some of the cellular and circuit level questions in combination with targeted genetic approaches we will be able to minimise the numbers of procedures and the burden on sentient animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined and developed our approaches over the past 15 years. The animals used in these studies will be acclimatised to the unit prior to any procedure and handled during this period to habituate them to human contact. Surgery will be performed aseptically under general anaesthesia with appropriate post-surgical analgesia. Animals will be closely monitored and allowed to fully recover after surgery before any subsequent intervention. The surgical procedures and implants for our chronic recording experiments have been refined over many years to minimise their severity and to reduce the numbers of animals required to meet our objectives. Animals will be group housed (whenever possible) with environmental enrichment. They will be monitored for signs of distress or excess weight loss and will immediately be removed from the study if these are detected.

We continually seek to refine our experimental approaches to keep up with the state of the art in our research field. We have been early adopters of new methods (such as optogenetics and chemogenetics) that have allowed improved, more powerful experimental designs using within animal comparisons and reduced need for implants. We have also introduced high density neuronal recording systems that has increased data yields and reduced the numbers of animals needed for experiment.
By monitoring spontaneous rather than evoked behaviour and using automated assessments based on video recording we have sought to both reduce stress and make behaviours more naturalistic. During the course of this project licence, we expect this process of continual refinement to be embedded in our thinking about experimental design and methodological improvement. We will consult with NVS/NACWO throughout the duration of the project and will regularly review the options for refinement of our procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidance to improve our reporting of research using animals, maximising information published and minimising unnecessary studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of the Physiological Society, the International Association for the Study of Pain, and Society for Neuroscience, which all have a clear focus on 3Rs and experimental approaches for the study of physiology and autonomic control. I attend and present at 3Rs events along with my research team and am informed by the regular circulation of information from the NC3R's and keep abreast of developments at www.nc3rs.org.uk. Within my institute I am part of a working group on in vivo experimental approaches in Neuroscience.

184. Regulatory Testing of Biological Medicinal Products

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Biological Medicinal Products, Regulatory Testing

Animal types	Life stages
Mice	adult, neonate
Guinea pigs	adult
Domestic fowl (Gallus gallus domesticus)	embryo
Rabbits	adult
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?

The overall aim of this project licence is to assess and confirm the safety of Biological Medicinal Products (BMPs) and to produce antiserum for regulatory purposes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is important to undertake this work in order that BMPs can be tested and guaranteed to be safe for general use. These BMPs are mainly produced by novel biotechnological processes and include naturally occurring human proteins and hormones (e.g. insulin required by diabetics), blood products (e.g. factor VIII required by haemophiliacs) and

cytokines (e.g. interferons used in the treatment of cancer). Novel viral vaccine antigens (e.g. Ebola and COVID-19) produced by recombinant DNA technology are also tested and all these products are undoubtedly having a profound impact across the entire medical spectrum. Many of these BMPs have added treatment options for diseases which previously had no treatment options. There are however potential safety concerns that arise from the novel processes used in their manufacture and from the complex structural and biological characteristics of the products. Such products therefore require robust testing by in-vitro and in-vivo methods to allow adequate assessment of safety. A risk based approach is commonly taken by regulators depending on the product itself and the manufacturing process. So it is essentially because the products are novel that the robust testing schedule is required (or at least until there is more historical data to confirm that a type of product or a production process is safe).

The protocols used for this work are refined and compliant with the relevant regulatory standards. Furthermore, there is an established track record of excellence in animal care and welfare for studies of this nature.

What outputs do you think you will see at the end of this project?

The primary output is the safe development and release of BMPs.

Who or what will benefit from these outputs, and how?

The short-term benefits from this output are directly related to ensuring the safety and availability of existing products. Each production batch of a BMP must satisfy a defined group of batch release criteria to ensure that the batch is safe and potent. Official batch release of BMPs always involves the review of batch protocols for compliance with the Marketing Authorisation (MA). This is particularly important for biologicals since they can be prone to variability in their production and testing and are administered under particular conditions. The failure of a batch to meet pre-defined criteria will lead to a dialogue with the manufacturer and result in a formal investigation intended to prevent the release of an ineffective or unsafe product onto the market.

The medium and longer-term benefits are primarily related to the testing of products in development such as cell banks and viral seed stocks (required by regulators as detailed under Project Plan).
Without this testing, the pipeline of new products would be severely depleted and would pose a major risk to human health, particularly considering recognised issues of resistance against antibiotics.

The potential impact of the outputs have clear potential to be highly significant in supporting the licensing of human proteins and hormones (e.g. insulin required by diabetics), blood products (e.g. factor VIII required by haemophiliacs) and cytokines (e.g. interferons used in the treatment of cancer and in particular a new BMP with dual mechanisms of action which directly attacks cancer cells and also stimulates the immune system to recognise and destroy cancer cells). Novel viral vaccine antigens (e.g. Ebola, COVID-19, HIV, HPV and Hep B) produced by recombinant DNA technology are also tested and all these products are undoubtedly having a profound impact across the entire medical spectrum. Many of these BMPs have added treatment options for diseases which previously had no treatment options.

This licence will play a key role in getting new BMPs into Stage 3 Clinical Trials and ultimately provide essential data for market authorisation.

How will you look to maximise the outputs of this work?

All the in-vivo tests offered by the company are specified in pharmacopoeial monographs, each with defined pass/fail criteria. A rigorous quality system helps maximise the outputs and for instance a robust and comprehensive 'Out of Specification' or OOS procedure is in place to investigate any failed or invalid tests. This OOS procedure runs in parallel with a 'Corrective and Preventative Action' or CAPA procedure. This dual system allows all information on product type tested (and media components) to be collated and referenced to prevent failed or invalid assays with similar product types. These systems also ensure that the testing service operates with very few repeats and the development and release of BMPs is not stalled or delayed.

Species and numbers of animals expected to be used

- Mice: Approx 6,000 per year
- Guinea pigs: Approx 300 per year
- Rabbits: Approx 24 per year
- Sheep: Approx 8 per year
- Domestic fowl (Gallus gallus domesticus): 50,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Viruses that do not cause cytopathic or other noticeable effects in a cell culture system may be detectable in an animal system. The following animal and life stages are efficient isolation systems for the following viruses:

Suckling mice - Arboviruses, Coxsackie A&B, Herpes Simplex 1&2, Rhabdoviruses, Togaviruses, Junin, Herpes B.

Adult mice - Rhabdoviruses, Togaviruses, LCM.

Adult guinea pigs - Rhabdoviruses, LCM, Lassa, Junin, Marburg, Ebola, Mycobacteria

Embryonated Hen's Eggs - Herpes Simplex 1&2, Rhabdoviruses, Herpes B, Mumps, Influenza, Parainfluenza 1,2&3, Vaccinia (embryonated eggs are also used for the detection of adventitious viruses, the procedures on these animals are carried out, out with the scope of the Act since they are undertaken before the animals are classed as protected, however monitoring of the animals may continue up to 17 days incubation.

The various regulatory guidelines such as EP dictate that testing of BMPs is required in protected animals as well as embryonated eggs. Although there is some crossover in the range of viruses detected in different species, the sensitivity is not uniform and this range of species is considered the lowest risk approach.
Adult rabbits (and occasionally sheep) are used for the production of polyclonal antisera which is used to comply with EP 2.6.16 which describes the in-vivo and in-vitro assays required to detect adventitious agents in viral vaccines for human use. Immunocompetent

adults mount an immune response to multiple epitopes on an immunising antigen and the resultant antiserum can successfully neutralise the majority of viruses.

Typically, what will be done to an animal used in your project?

The vast majority of test articles will contain no adventitious viruses and there will be no adverse effects in the animals. In addition to daily general health observations throughout the study as part of routine husbandry procedures, all animals will be clinically monitored by an experienced person on a daily basis for up to 42 days following inoculation. Any animal exhibiting more than moderate discomfort or loss of condition will be euthanased on humane grounds and a necropsy may be carried out.

There is an expected mortality (<0.01%) for adult mice and guinea pigs. These unexpected deaths can occur occasionally and are often not related to test material. For antiserum production, an animal may be immunised on up to four occasions with adjuvanted antigen. The duration of these studies is normally 56 days and over that time up to four blood samples may be collected. Animals are exsanguinated under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects in safety studies are few and infrequent therefore >99.9% of animals will complete a study without experiencing pain or discomfort except at the time of administration. The occasional animal experiencing pain or discomfort is normally due to toxicity of a medicinal product which the manufacturer was not anticipating. Animals are always euthanased if in any distress or at the end of a study by an approved method.

Adverse effects in antiserum production studies are minimal and all animals should complete a study without experiencing pain or discomfort except at the time of administration or 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 animal type)?

Suckling mice (adventitious agents) - Expected severity is MILD and expectation is 100% but experience is that ~0.01% of animals have exceeded this annually.

Adult mice (adventitious agents) - Expected severity is MILD and expectation is 100% but experience is that ~0.01% of animals have exceeded this annually.

Adult guinea pigs (adventitious agents) - Expected severity is mild and expectation is 100%.

Rabbits (antiserum production) - Expected severity is mild and expectation is 100%.
Sheep (antiserum production) - Expected severity is mild and expectation is 100%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For adventitious agents testing, the safety of new products is being evaluated in animals to comply with regulatory requirements. It is therefore not possible to move towards in-vitro alternatives without approval from governmental regulatory bodies - essentially national and international legislation would have to change.

Which non-animal alternatives did you consider for use in this project?

The use of non-animal alternatives have not been considered for adventitious agents testing for the reason stated above. The use of non-animal-derived antibody technologies have however developed considerably in recent years and clients are encouraged to develop non-animal alternatives through the period when a product is being developed (particularly later stages). Due to complexities relating to the validation of the neutralisation process, this can take considerable time and animal derived antibody is required in the early stages of development. Antiserum production services are not offered for licensed products.

Why were they not suitable?

The use of non-animal derived antibody has been assessed by clients* and considered problematic because often they cannot recapitulate the properties of animal-derived antibody and for instance they may neutralise and impact the detection of other similar viruses and is therefore considered high risk in terms of human safety. That said, generation of system-compatible, neutralising synthetic antibody is technically possible but requires a framework with diversity restricted complementarity-determining regions which is a lengthy process. This should be possible for licensed products but is not feasible when a product is in development. Going forward, the production of neutralising antibody in animals will only be offered for a product in the early stages of development or if neutralisation by synthetic antibody is impossible.

*We were a strategic partner in the release of a COVID-19 vaccine and antiserum was required to neutralise the vaccine's viral vector. In this instance, the use of commercial antibody was not feasible since there was a risk that it neutralised and impacted the detection of other potential adventitious adenoviruses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

Numbers have been informed by experience under predecessor licences.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Group sizes are set in the various regulatory guidelines and the criteria for test pass or fail are clearly defined in the pharmacopoeial monographs.

Animal studies will only be conducted when there is a reasoned, sustainable justification for the generation of new test data, there is no validated alternative to animal testing and the protocol will generate data which will meet the specified objective, will be scientifically valid and will be acceptable to the relevant regulatory authorities.

Eggs numbers listed are for eggs which will be incubated for up to 17 days.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Steps are taken to ensure that the number of control groups is minimised by running several test groups beside a single control group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For the detection of adventitious agents, models and methods are set in the various pharmacopoeial monographs.

For the production of polyclonal antiserum a 'double emulsification' procedure is used prior to immunisation to ensure that any local reactions/response to immunisation is minimal. This procedure was developed within the Establishment and positively improves the experience of the animal without compromising the quality of the antibody.

Why can’t you use animals that are less sentient?

For the detection of adventitious agents, life stages and species are set in the various pharmacopoeial monographs.

For the production of polyclonal antiserum, mature animals with fully developed immune systems are required. The final exsunguination is undertaken under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Care is always taken to ensure that increased monitoring is in place if any animals show signs of discomfort or distress (or where it might be anticipated). Veterinary support is also available 24/7 should the need be required.

The provision of environmental enrichment is also a priority for all studies. For example all antiserum production studies in recent years have employed communal floor pens where rabbits have raised areas, tunnels and other items. As a result of the work of the 3R's committee the Establishment has introduced enriched and improved environments for avian species, goats, cattle and also introduced a new welfare-friendly farrowing crate system.

At each meeting of the Establishment AWERB, in addition to other business, specified project licensees, or their representatives are asked to present details of experiments or project licence applications or amendments with particular reference to their continued justification for animal usage. The members of the AWERB are invited to give their comments and advice for any amendments required, with particular relevance to the application of the 3Rs. This licence is therefore under the constant scrutiny of the AWERB.

Refinements and improvements are shared with other licence holders working in similar areas.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Establishment 3Rs committee produces newsletters and presentations on housing and enrichment for staff working directly or indirectly with experimental animals.

The NC3Rs website is used for guidance and information and this website also provides links to other relevant publications and events.

European Commission reports are also circulated and discussed e.g. EURL ECVAM Status Report (2020) - Non-animal Methods in Science and Regulation and EURL ECVAM Recommendation on Non- Animal-Derived Antibodies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Establishment has a 3Rs committee and one person from the team involved with this licence sits on this committee. There are regular communications and training recommendations from this committee to all personal and project licence holders. The main objectives of the 3Rs committee is as follows:

To provide a framework of people across all departments with an interest and experience in the 3Rs.

To produce a resource of techniques and materials to facilitate Enrichment and Refinement wherever possible.

To provide a vehicle that supports engagement with enrichment and refinement during the ethical review process to include reporting on and discussion of methods employed.

185. Profiling of test agents in rodents in a drug discovery platform

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pharmacokinetics, Drug discovery, Tolerability, Biomarkers, Safety

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to generate data on the pharmacokinetics, pharmacodynamics and tolerability of test agents for the disease areas listed in this licence, and the effect of test agents in rodents over a longer period of dosing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 crucial data in the preclinical (before human dosing) development of potential new drugs for the treatment of cancer, cardiovascular diseases and disorders of haemostasis, metabolic diseases, inflammatory diseases and neurological diseases. All of the diseases we work on are diseases were current treatments are not available or need improving upon. These studies will generate important information that

cannot be found without the use of animals. All clients that we work with are developing test agents (potential new drugs) which will hopefully contribute to helping people with various diseases where current treatments are not available or need improving upon.

This project investigates both how the test agents are processed and removed by the body (pharmacokinetics, or PK), and how the test agents affect the function of the body (pharmacodynamics, or PD). This requires information from a whole animal system and cannot be replicated in a cell or a test tube. The project will determine important characteristics of test agents to decide the best dose levels, dose routes (how the drug is taken) and patterns of dosing (how often the drug is taken), whether the test agents have early signs of unwanted side effects, and whether the test agents are likely to work as an effective treatment for disease. This information will be key in decisions regarding which test agents should continue to be developed, and which are unsuitable for further work. Ultimately, this will feed into which test agents make it into clinical trials, where they are tested in humans. Our approach to the work ensures that this is done in the most efficient way possible, and that the use of every animal is maximised.

What outputs do you think you will see at the end of this project?

As part of this project, we expect to test the PK (what the body does to the drug) of at least 30 test agents, of which the majority will be new test agents. We expect at least 10 of these to move onto further work either under this project or other projects. In addition, we expect to run a minimum of 20 longer term studies looking at the safety and tolerability of test agents as well as how they change body function as part of this project.

Data produced by studies under this project may be used to support patent and funding applications by clients. They will also support the design of regulatory preclinical (animal) studies for clients and may be included in applications for regulatory approval. We will also provide valuable information from this project that will halt the further development of several test agents, preventing unnecessary use of animals in studies using test agents that would not be suitable for use in humans. These test agents will be for potential use as a therapeutic in the following disease areas:

Cancer

Cardiovascular diseases

Disorders of haemostasis

Metabolic diseases (including inborn errors of metabolism such as alpha-1 antitrypsin deficiency)

Inflammatory diseases

Neurological diseases

Who or what will benefit from these outputs, and how?

This project will generate important data in the development of potential new drugs.

The PK analysis which determines how much drug gets into the body and what the body does to the drug will enable our clients to select the best test agents (drugs) for further development. These studies show how long it takes for drugs to get into the blood and the

length of time they stay in the blood or tissues (parts of the body) before the body gets rid of them. As well as providing information for key decisions as to whether test agents are likely to make suitable drugs, PK studies provide vital information required to design the longer-term studies needed in drug development. Ultimately, PK studies also feed into the design of clinical trials.

The safety and tolerability work will check that test agents are suitable for repeat dosing over longer time periods and look to see if test agents have any early side effects. These studies will help with working out the best dose for potentially treating disease. They also make sure that we identify any unwanted side effects that mean that they would not be suitable for use as a drug for humans. These studies also help us to design longer term studies in the best way possible to provide the information required about the way the test agent is working.

The longer-term dosing studies in this project will allow us to investigate the effects of the test agents on biomarkers, which are measurable levels of key molecules in the body which tell us whether body function has been altered by the drug. This will provide us with information on whether the test agents are able to alter the levels of biomarkers that are most relevant to the disease that the test agent is designed to treat. If a test agent does not alter the important biomarkers, this may mean it will not be a suitable treatment for disease.

Our focus on using a science-led approach will enable key decisions to be made at each development stage on whether a test agent is likely to become a successful drug. This allows test agents which are not suitable as drugs to be abandoned at an early stage using the fewest number of animals possible for each test agent. The identification of test agents as unsuitable for use in humans at an early stage of development will also ensure a better success rate in the drug discovery process than has previously been seen in the pharmaceutical industry. Ultimately, this project will contribute to the successful development of new drugs, which will benefit patients with the diseases being treated.

Health conditions linked to inflammation are very common and half of deaths worldwide can be linked to diseases involving inflammation. Cardiovascular and metabolic diseases lead to a third of deaths worldwide. Disorders of haemostasis contribute to these deaths where abnormal clotting occurs. In the case of the rarer diseases where clotting is reduced, such as Haemophilia, the diseases reduce life expectancy. Cancer is also sadly a leading cause of death worldwide, with more than a quarter of deaths in the UK involving cancer. With both an aging population and the increasing problems with obesity in society, the occurrence of these diseases is likely to increase. Neurological diseases are also increasing, with Alzheimer's and dementia the leading cause of death in the UK in 2018. There is a lot of overlap between the diseases covered by this project, for example a rise in cardiovascular and metabolic diseases such as heart disease and diabetes increase the risk of stroke and the associated life-changing neurodegeneration. Therefore, it is clear that investigation into potential new drugs for all of these diseases is required to improve chances of survival, but also to improve the quality of life for patients living with the diseases.

How will you look to maximise the outputs of this work?
All studies are designed such that the outputs from each animal are maximised. Expert knowledge is gathered not only from within the preclinical (animal work) team performing the animal studies, but from other teams at our company. This ensures that all relevant work that has been performed in the laboratory is taken into consideration when designing the animal studies. The *in vitro* (in the dish/test tube) and bioanalysis teams at our

company are experts at analysing tissue and blood samples collected from animals, and they help with details of sample collection and storage to ensure that the samples are collected and stored in the best way possible. They are also experts at working with small samples, particularly small blood samples, meaning that they can often analyse lots of different biomarkers and test agent levels from each animal.

In addition, we will seek expertise from our established networks both within our establishment and further afield, to ensure that we make use of any new knowledge or better methods of performing animal studies. We will also use these networks to give information to others about any ways in which we can help via our research. This will include sharing information about unsuccessful approaches in addition to sharing information on any refinements and improvements. We will maintain good communication with managers of the animal facilities to ensure that any tissues from animals being killed that are not required for our work can be made available to other researchers.

Due to the nature of the work undertaken in this project we are unlikely to be able to publish data, as this would put ourselves or our clients at a competitive disadvantage. However, where advances are made in study design, we will publish or share these wherever possible.

Species and numbers of animals expected to be used

- Mice: 3200
- Rats: 1750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Both mice and rats are well established 'models' for use in studies determining the PK and tolerability of new test agents and the effect of test agents on biomarkers. This is due to their similarities to humans, with these 'models' mimicking aspects of human physiology and disease. They are very well characterised, meaning that there is a lot known about how the body works in mice and rats, both when healthy and with diseases. This helps in the interpretation of results from studies using test agents. We are using adult animals in this project as this will reduce the variability in the results and is the life stage most relevant to the majority of test agents. This project will look at test agents for the following disease areas:

Cancer

Cardiovascular diseases
Disorders of haemostasis

Metabolic diseases (including inborn errors of metabolism such as alpha-1 antitrypsin deficiency)

Inflammatory diseases

Neurological diseases

Typically, what will be done to an animal used in your project?

In the majority of studies, test agents will be administered to animals. This dosing will be by a number of different methods. Test agents will be administered intravenously (into a vein), intraperitoneally (into the abdominal body cavity), subcutaneously (under the skin), orally (either using a dosing tube or via adding the test agent to the food or drinking water), intranasally (inhaled into the nose), intracolonically (into the colon) and/or intraduodenally (into the small intestine). Where dosing is performed intranasally or intracolonically, this will be in animals who are anaesthetised. Intravenous injection may also be performed under anaesthesia but will usually be performed in conscious (not anaesthetised) animals.

On occasions a slow-release device will be implanted for the subcutaneous or intraperitoneal administration of drugs.

In PK studies animals will usually be given one dose of test agent and blood samples will be collected multiple times after this dose is given. In some PK studies, we will use animals with a cannula, which is tubing allowing direct access to either a blood vessel or to the gut. These cannulae require surgery to put them in place. This surgery will either be performed by scientists at our company using our in-house expertise, or more commonly performed by an external supplier who are experts in these surgeries.

When using animals with a cannula into a blood vessel, they may be given the same test agent twice, once straight into the blood (intravenous) and once by a different route, with blood samples taken after both doses. There will be enough time between the two doses to make sure that no test agent is left in the body before the second dose. This will allow calculation of how much of the drug given by an extravascular route (not straight into the blood, for example given orally) makes it into the blood.
For safety and tolerability studies, animals will be dosed with test agent(s) over a period of several days or weeks. In some safety studies the dosage will be set at a level predicted to be effective when used as a treatment in animals to check if any side effects are observed. For tolerability studies, the concentration of test agent(s) may be increased during the study to test if higher doses have early side effects. Blood samples may be collected during the study and blood and tissue samples will be collected at the end of the study to make sure as much data as possible is generated from each animal. During safety and tolerability studies, blood samples may also be taken to investigate the PK of the test agents being used, and whether this is changed after the animals have received the test agent for several days.

For dosing studies, animals will be dosed with test agent(s) over a period of several weeks or months. Dosing will usually be daily, but this may vary depending upon the test agent. Blood samples may be collected at regular intervals during the study for the measurement of biomarkers or test agent levels. Other non-regulated procedures such as urine collection may also be performed. Blood and tissue samples will be collected at the end of the study to maximise the data generated from each animal.

Where animals are used only for the collection of blood or tissue samples for *ex vivo* work (work outside of a living body in the laboratory), all procedures will be performed under non-recovery anaesthesia where possible, such that the only procedure the animal experiences is the process of being anesthetised. They will then be kept unconscious under deep anaesthesia whilst blood and/or tissue(s) are collected. On some occasions, animals may be dosed with a substance prior to humane killing, if required for the *ex vivo*

work. Again, where possible this will be performed under non-recovery anaesthesia, but on some occasions it may be necessary to dose animals over a period of weeks prior to blood collection. Terminal blood and tissue samples will be collected immediately before death and rodents humanely killed. Additional tissue samples may be collected after death.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals used for PK studies, longer-term dosing studies and those used for the collection of blood samples for *ex vivo* work should not experience more than transient (short-lasting) pain and discomfort. Those animals with a cannula for PK studies will go through a surgery before the PK study is performed. The surgery will be performed under general anaesthesia, such that the animal will remain in a state of unconsciousness throughout the surgery. Recovery from surgery is expected to be quick, with recovery from anaesthesia within 2 hours. In most cases, the cannulation surgery will be performed by an external supplier who have extensive expertise in these surgeries and reliably perform the surgery without problems. Animals will be allowed to recover for a suitable period determined by the supplier (currently 7 days) prior to shipment to us. For vascular cannulation, we often use vascular access buttons located in the interscapular region. These vascular access buttons connect to the end of the cannula and have a magnetic metal cap to cover the access point, meaning that animals are able to be group housed without the risk of the cannula being damaged by cage mates. In all cases the cannulae do not cause problems with the ability of the animal to move freely and scratching at the cannula site is only observed on very rare occasions. Where a slow-release device is used in longer- term dosing studies, these animals will undergo surgery under general anaesthesia, with quick recovery expected. The location of the slow-release device is not expected to have any effect on the animal's ability to move freely.

The majority of animals undergoing safety and tolerability studies will only experience transient pain and discomfort. However, some animals may experience more significant side effects. These could include weight loss (up to 15%, or 20% when using test agents designed for the treatment of cancer), pain and subdued behaviour. There are two main reasons when these studies may be required to cause more side effects. One of these is where the test agents are designed to treat fatal human diseases, where more side effects are acceptable in humans, for example in the case of chemotherapy for treatment of cancer. The other occasions are studies where it is important to find out what the maximum dose that can be given to animals is. Understanding the maximum dose that can be given is important as it allows decisions to be made on the doses for all future work. In these studies the dosage will be gradually increased until side effects are noticed. Once side effects are observed the dose will be held at that level and the study typically completed within 72 hours. This allows us to see if the animals develop tolerance to the drug and the side effects normalise, or whether the side effects are sustained. Animals will be closely monitored to ensure that they do not suffer excessively.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse - Mild: 66% Mouse - Moderate: 26%
Mouse - Non-recovery: 8% Rat - Mild: 59%
Rat - Moderate: 30% Rat - Non-recovery: 11%

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 work to be performed in this project cannot be fully replaced by using models in a laboratory. Wherever possible, work is performed either using cells or tissues taken from an animal before moving into animal studies, to make sure that the animal studies are well designed and that the maximum amount of data is gained from each animal study. However, this project aims to investigate the PK, tolerability and effect on biomarkers after dosing with test agents, which cannot be determined without using animals.

Which non-animal alternatives did you consider for use in this project?

Work can be performed in the laboratory to ensure that test agents are acting as expected before moving into animal studies. These may be using cell lines grown in the lab, or using tissues collected from animals. For example, we have previously performed these types of experiments by running assays (laboratory experiments) looking at how quickly blood clots form in rodent blood, or by looking at the effect of a test agent on liver cells collected from a mouse. We use these studies before moving into animals to make sure that test agents that are unlikely to be useful to humans do not get tested in animals.

Why were they not suitable?

The use of animals is essential to determine how the body acts on test agents, and how test agents act on the body. It is not possible to determine how a drug gets into the blood and how it is broken down and removed from the body without using live animals. Similarly, it is not possible to determine whether a test agent will have side effects using cells or tissues in a laboratory, or to find out the effect a test agent will have on biomarkers.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used has been estimated by analysis of the number of animals used on previous projects, looking at the number of animals required for each type of study. This was then combined with a prediction of likely demand of future projects to give the numbers in this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have extensive experience in the design of experiments of the types in this project, which has given us confidence in the number of animals required to ensure that no animals are used unnecessarily, but also that the data generated is reliable. Whilst through experience we do tend to use a standard group size for the majority of our pharmacokinetic (3 per group) and tolerability (5 per group) studies, we regularly refer to the PREPARE and ARRIVE guidelines and make use of the NC3Rs Experimental Design Assistant to ensure that we are using the correct number of animals for every study. When designing experiments for the effect of test agents on biomarkers, we also look at published literature to determine the variability observed in the biomarkers of interest where we have not tested these before. Where information is not available in published literature or from contact with other researchers, pilot studies in a small number of animals will be used first where appropriate to assess the variability of the biomarker to be tested and to determine the appropriate group size for future experiments. We can then use the NC3Rs Experimental Design Assistant to help determine the most appropriate group size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Data from pilot studies and previous experience are used to ensure that the numbers used are both as low as possible, but also large enough to generate reliable data. Within our company, a member of the wider team has generated a tool for performing power calculations and can be consulted as necessary to assist with study design.

Where genetically altered animals are required, these will usually be provided by our breeding project, which will ensure that animals are bred efficiently using as few animals as possible by communicating need with colony managers. Where animals are obtained from external sources, only the number of animals required for the study will be purchased or imported.

Wherever possible, our *in vivo* (animal work) scientists will be blinded to the treatment status of an animal, reducing bias. This enables more reliable information to be gathered from a smaller number of animals. Those who carry out analysis on blood and tissues collected during the study are also blind to any treatment with test agent where possible.

Baseline data (e.g. bodyweight, biomarker levels) are recorded and animals assigned to treatment groups to minimise differences between the groups at the start of the study.

Good planning ensures that within any series of studies we can control for variability that might be introduced. To limit this variability we look at using animals of a similar age/weight range, testing different batches of test agent in the lab first, using the same source of reagents (chemicals used during the experiments), keeping records of observations made and standardising as many components of the *in vivo* model as is practicable.

We will also provide valuable information from this project that will halt the further development of several test agents, preventing unnecessary use of animals in studies using test agents that would not be suitable for use in 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.

Which animal models and methods 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 both mice and rats during this project. Some of these will have a genetic alteration that will not cause them harm, but which provides a more appropriate model in which to perform experiments on test agents for particular diseases.

PK studies are generally performed using a low dose of the test agent which reduces the chance of any unwanted side effects. However, when the test agent has not been dosed to animals previously, a small group will be dosed and monitored for 2 hours to ensure that there are no side effects before the test agent is given to the remaining animals. These animals will be further monitored up to 6 hours post dosing to ensure any side effects that occur several hours after dosing are also observed. PK studies involve taking blood at several different periods of time after dosing (time points) from as short as 2 minutes to as long as days after dosing. Where multiple animals are dosed with the same test agent, blood samples will be collected at different time points from the different groups of animals. The animals dosed first will be those for longer time points, with animals for shorter time points dosed subsequently. This means that side effects which are not seen until more than 2 hours have passed will not be relevant to animals dosed later in the day and that it is appropriate to begin dosing additional groups after only 2 hours. If any side effects are observed, the study would be stopped or the dose lowered. If side effects are observed, these would likely be subdued behaviour, piloerection (hair standing on end) or a hunched appearance.

Where it is hard to predict the potential side effects of a test agent, studies will be performed in a small number of animals first who will be monitored for at least 2 hours or overnight in the case of test agents where side effects are likely to take longer to be seen. Where side effects are observed, the dose may be altered to ensure no long-lasting side effects are seen before any longer-term studies are performed.

Dosing will always be performed using the least invasive route (e.g. oral dosing via food or water if possible), and where a needle is required using the smallest needle possible.

Why can’t you use animals that are less sentient?

Mature mice and rats are essential for studying complex diseases, where all systems must be similar enough to humans to find the most effective drugs. The duration of most studies in this project are longer than can be performed under anaesthesia as they span several days or weeks. The anaesthetic drugs themselves may also affect the results of the studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have a demonstrable history of refining our procedures and practices to minimise harm to our experimental animals. For example, we always use the smallest needle possible for dosing to minimise any pain and distress to the animal. We also investigate the suitability

of test agents administered orally to be dosed via the drinking water rather than via oral dosing, where the dose is administered via insertion of a tube down the throat. We recently implemented this for a test agent, allowing us to switch from twice daily oral dosing to dosing via the drinking water. We will also sometimes use a slow-release device for dosing. Whilst this requires an initial surgical procedure, it reduces the need for the stress of regular injections.

During studies that involve repeat dosing, animal condition and bodyweight is monitored regularly, with body weight recorded prior to each dose as a minimum. We are developing a scoring system to monitor any side effects observed due to dosing, which will help us to ensure that these are tracked and acted upon as necessary.

Animals will be housed in social groups in the vast majority of cases, with animals housed alone only where this is absolutely necessary (for example in some animals who have a cannula inserted, where cage mates may dislodge the cannula). Where anaesthesia is used, care will be given to support animals during anaesthesia and recovery, using heat hats to maintain body temperature and providing soft diet and hydrogel to aid recovery as appropriate.

We use microvettes (small capillary blood collection tubes, coated with anticoagulant if appropriate to prevent blood from clotting) to collect small blood samples from animals. This allows us to take small samples in the most efficient way possible. We have also developed methods to be able to use a mixture of blood and water for some of the tests run on rodent blood, which allows us to collect a smaller volume of blood than would be required when using standard methods of preparing plasma from collected blood.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be using PREPARE guidelines for the planning of studies and follow the latest version (2020) of the ARRIVE guidelines for ultimate reporting of data. The LASA document ‘Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals’ will be used when planning studies, to help guide study design. This document, particularly the section detailing the report of the FELASA working group on pain and distress has also been used when setting the humane endpoints within this project. Where surgery is performed, we will refer to the LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. We will also regularly check the current list of LASA publications, to ensure that any relevant to work under this project are taken into consideration.

Similarly, we will regularly reference the NC3Rs to ensure that we are implementing best practice for the techniques undertaken.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

When designing animal studies we consider the appropriate guidelines, including the guidance from LASA, the NC3Rs and the PREPARE and ARRIVE guidelines. This guidance will influence our study design. One example of this is the use of the NC3Rs guidance on the number and volume of bleeds to be taken from rodents in the design of pharmacokinetic studies. We regularly check the NC3Rs website, along with other external resources such as Norecopa and Jax, to ensure that we are using the most refined methods and are aware of any improvements to procedures that have been developed.

186. Vascular cytoprotection during inflammation

Project duration

0 years 8 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

angiogenesis, cardiovascular disease, endothelial homeostasis, inflammation, endothelial transcription

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of our work is to further our understanding of the transcriptional pathways which regulate endothelial homeostatic and protective genes, and modulate the complex process of angiogenesis. The ultimate long-term aim is to define the means to therapeutically target these pathways to: (i) prevent and reverse endothelial dysfunction and atherogenesis, and (ii) specifically enhance or inhibit angiogenesis as required in different disease scenarios.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work? Cardiovascular Disease
Cardiovascular disease, and atherosclerosis (which is the build up of fatty substances in the walls of blood vessels leading to restriction of blood flow to vital organs) in particular,

remains the biggest killer in the developed world and are increasingly common in the emerging economies. Despite this, preventative strategies are relatively ineffective and treatment is often delayed and only initiated after presentation with angina, myocardial infarction or stroke. This in turn results in a huge financial burden to health services. Critically, therapeutic options remain limited and progress has stalled since the introduction of statin therapy. Thus an important challenge remains, namely the development of optimal preventative treatment. We propose that improved understanding of the biology of the vascular endothelium, the single layer of cells that line the surface of all blood vessels, will allow us to identify novel therapeutic targets and ultimately novel therapies aimed at preventing or retarding cardiovascular disease.

Vascular injury and endothelial dysfunction

Vascular endothelial cells (EC), which line all blood vessels, play a central role in the regulation of inflammation, vascular tone, blood clotting (haemostasis), wound healing and new blood vessel formation (angiogenesis). By virtue of its anatomic location the vascular endothelium is continuously exposed to harmful factors including inflammatory mediators circulating in the blood, white blood cells (leukocytes) and activated forms of a cholesterol transporting protein (oxidatively-modified LDL).

Moreover, endothelial injury and dysfunction may contribute to the premature atherosclerosis seen in patients with diabetes, renal failure, systemic lupus erythematosus or rheumatoid arthritis. In order to combat this, cytoprotective pathways have evolved which are activated during inflammatory responses. They act to maintain vascular integrity and assist endothelial repair. An important additional function of the endothelium is angiogenesis, the development of new blood vessels from pre-existing vasculature. This process plays an essential role in vascular repair, wound healing and remodelling.

However, aberrant angiogenesis has also been implicated in a variety of diseases including cancer, rheumatoid arthritis and atherosclerosis. Vascular endothelial injury is the earliest detectable event in atherogenesis and predisposes to endothelial dysfunction. Our initial approach has been to study the molecular mechanisms involved in endothelial homeostasis, vasculoprotection and angiogenesis, with the ultimate aim of therapeutic manipulation. Endothelial dysfunction is characterised by a local inflammatory response, impaired nitric oxide biosynthesis, enhanced oxidative stress and increased permeability to lipids and monocytes. If unchecked this dysfunction leads to endothelial injury and programmed EC death (apoptosis), which occurs preferentially at arterial branches and curvatures, where loss of arterial endothelium and exposure of the underlying extracellular matrix enhance the risk of thrombosis.

What outputs do you think you will see at the end of this project?

The predominant output during the duration of the licence will be in the form of novel scientific data published in peer-reviewed scientific journals with an open-access platform. We will also continue our regular presentations at Local, National and International conferences including the European Vascular Biology Organisation seminars and International Vascular Biology Meetings. We will also engage in meetings aimed at presenting scientific data to the public which are organised by my institution, and funder-supported outreach events and site visits. These data will be available to other researchers both scientific and clinical.

Who or what will benefit from these outputs, and how?

We believe that therapies are required that act to reduce the incidence of atherosclerosis-related disease in those patients known to be at high risk. For example those with diabetes or inflammatory diseases. Our focus is endothelial dysfunction and how we might reverse that. We believe that we have identified pathways that are important for endothelial protection. The current project will seek safe and effective ways to switch the protective pathways on and so enhance endothelial function.
Ultimately if drugs can be developed to mimic these responses then patients shown to have endothelial dysfunction might benefit from these therapies.

How will you look to maximise the outputs of this work?

Our aim in the medium and long-term will be to translate the findings in animals in to human studies and then, either independently or in collaboration with industry. This will be a new step for our group and we will approach our institution's innovations department at this point for help and advice concerning interactions with industry. This will also include attending joint meetings arranged by our institution with potential commercial partners. Our aim will be to develop small molecules that target important endothelial signalling pathways and to test their efficacy as therapeutics. Currently we are particularly focused upon protective pathways regulated by endothelial expressed protein kinases and endothelial transcription factors.

Species and numbers of animals expected to be used

- Mice: 700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using adult mice for the experimental parts of our project as they are widely available in the genetic form we require and they have a well-researched and understood immune system. There are no other animals that serve this purpose as well as mice. For this project, we will be using mice (with or without associated wild-type controls) that are genetically modified to either inactivate or turn on different genes so that we can study how these genes control important processes such as the body's response to inflammation or how new blood vessels form (a process called angiogenesis). In some instances, mice will also be given a high fat diet in order to study the development of fatty substances or plaques in the blood vessel walls (known as atherosclerosis). The use of these models will however be kept under review, and we will consider refining our approach by moving to an even more informative and clinically relevant model if one becomes available.

Typically, what will be done to an animal used in your project?

Animals may be administered substances such as drugs, proteins, antibodies, nanoparticles, genes or other agents for up to 16 weeks, in order to modify specific outcomes, such as to induce changes in gene expression. This will be carried out using injection of substances or through the use of osmotic pumps or a slow release pellet, or by incorporating substances into the animal's food or water. Animals may subsequently be fed a high fat diet for periods of up to 4 months. Mice may be injected with substances in

order to induce inflammation or we may inject them with a basement membrane matrix (e.g. Matrigel) in order to study how newly formed blood vessels are affected by changes in gene expression or dietary modification. Performing studies using genetically modified mice will allow us to study the effect of the progression and regression of disease using these models.

Blood sampling may occur at certain stages.

Animals may be injected with targeting agents for imaging. Under general anaesthesia animals may have their hair removed using shaver and special creams, and then be scanned using positron emission tomography (PET), ultrasound or optical imaging, computed tomography (CT), or magnetic resonance imaging (MRI).

What are the expected impacts and/or adverse effects for the animals during your project?

All expected impacts or adverse effects (such as ruffled fur, hunched posture, reluctance to feed or move, weight loss >20%) will be monitored carefully by a licensed member of our team alongside a named veterinary surgeon and named animal care and welfare officer.

Potential adverse effects include: skin damage from high fat diet, infection from administration of treatments, blood loss from blood sampling. However we will carefully monitor these and where possible reduce these impacts.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All mice used within this license are expected to fall under the mild (~70 %) or moderate (~30%) severity categories.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

A detailed understanding of the cellular mechanisms underlying endothelial homeostasis, vascular protection against injury and regeneration will lead to novel therapies for human disease. Where possible, our laboratory performs experiments *in vitro*, using cultured human endothelial cell (EC), to replace *in vivo* studies. However, the complex nature of blood vessels cannot be fully recapitulated *in vitro* and it may be necessary for some experiments (such as *in vivo* analysis of vessel development) to be carried out using murine models. These assays are well established in the group with clear end points and

will be used to test the hypotheses generated by the detailed in vitro experiments. In this way we will minimise the number of animals required and any associated suffering.

Which non-animal alternatives did you consider for use in this project?

We are constantly seeking non-animal models in the published scientific literature, we attend relevant scientific conferences and we discuss alternative options with collaborators. In addition we have searched PubMed and EURL-ECVAM databases. We have established an *in vitro* flow apparatus to model the impact of shear stress exerted by blood on human endothelium. We are developing 3D organ-on-a-chip and microfluidic angiogenesis models with human cells. We have perfected methods for the isolation and culture of late outgrowth endothelial colony forming cells (ECFC) from human blood. We have generated conditionally-immortalised EC lines from genetically engineered animals to minimise animal usage.

Why were they not suitable?

Chronic inflammation, endothelial dysfunction, angiogenesis and vascular injury are complex multicellular responses that cannot fully be modelled in vitro. At the present time, *in vivo* models are the only definitive way of studying the efficacy of novel therapies and testing hypotheses generated from *in vitro* 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?

Over 10 years we have developed protocols to minimise animal numbers required, while ensuring with statistical advice that rigour is maintained.

Principles of experimental design and data analysis.

We use detailed *in vitro* analysis to frame a clear question and then plan the key *in vivo* experiments:
As part of experimental planning we use guidance from our previous experiments and/or those in the literature.
To minimise variation we use matched littermate controls.

To randomise, animals are sequentially allocated from single cages to test or control groups.

For these established protocols we aim to achieve 80-90% power, $p<0.05$, and 25-50% differences, and use the analysis of variance statistical analysis with a post-hoc test if required. From our previous experience we expect that groups of 6-8 animals per treatment group should be sufficient to obtain the required results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The models of inflammatory disease or angiogenesis we have developed are extremely reliable and reproducible, so we can accurately calculate the numbers of mice required for each treatment group in order to obtain scientifically significant results from small groups of animals. We combine experiments where possible to make maximum use of controls and so further limit experimental variation. We have also developed approaches to maximise use of tissues from each animal, for example by collecting multiple tissues after death. This ensures that we get as much data as possible from each mouse which thereby limits the number of mice needed.

We have designed the experiments and the methods of analysis to allow the results to be published in accordance with the NC3Rs' ARRIVE guidelines (Kilkenny et al. PLOS Biol 8(6): e1000412).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Further aspects of experimental design to ensure the minimum number of animals:

Use of differentially labelled antibodies which allows data on different molecules to be obtained from single tissue specimens.

Introduction of non-invasive imaging strategies which allow accurate longitudinal analysis, so reducing animal numbers.

Harvesting and storage of post-mortem tissues allows subsequent use in different experiments/ projects, so optimising data output and reducing the total number of animals required.

To maximise data output from the sub-cutaneous air pouch model, above and beyond analysis of leukocyte migration in to the pouch, we dissect out the pouch tissue at the end of the experiment and prepare RNA from this. We can then analyse changes in adhesion molecule expression in the blood vessels lining the pouch. In parallel some tissue is conserved for subsequent comparative confocal microscopy analysis. This has increased the data obtained and optimised interpretation of the mechanism underlying the changes in leukocyte trafficking

We use freezing of embryos to avoid unnecessary breeding of genetically-modified mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have proved invaluable for our studies, largely due to the availability of genetically-engineered strains to model human disease. For example, LdlR knockout mice represent a good model for human atherosclerosis with minimal animal suffering.

Genetic manipulation or specific molecular targeting of signalling molecules allows specific analysis of the role of these molecules without needing to treat animals with pharmacological inhibitors, which are less specific and often toxic.

Our protocols have been devised and refined so that they are all mild or moderate. Examples include the subcutaneous air pouch and peritonitis models of inflammation and the Matrigel model of angiogenesis. The local models of inflammation e.g. sub-cutaneous air-pouch and peritonitis have minimal impact and do not require analgesia in contrast to the foot pad model which we no longer employ. These two models have also reduced our use of systemic inflammatory models.

Why can’t you use animals that are less sentient?

Mouse models of atherosclerosis or for inflammation research are common and well validated. There are no high fidelity animal models for these processes in less sentient animals. Larger animal models (such as pigs or rabbits) are well validated, but are even more sentient than mice. Therefore, using mice is the best possible option for exploring experimental models of atherosclerosis, inflammation or angiogenesis in high fidelity models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our procedures are all reviewed by ourselves and by NACWO and NVS to ensure we are minimising animal welfare harms. Where recovery from surgery is required, the animals are recovered in a warmed cabinet and analgesia administered as standard following advice of the local NACWO and NVS. Animals asleep under general anaesthesia have the depth of anaesthesia monitored closely to ensure appropriate level of sleep. This is performed by checking toe pinch reflexes as well as respiratory rate monitoring and general inspection. Following any procedure with the potential to cause harm, our team and the technicians that closely work alongside the animals, regularly monitor the mice to check for signs of ill-health, pain or distress and poor welfare.

We regularly consult the NC3Rs website to see whether any techniques that we are using can be refined. We will also continue to use the general approaches we have demonstrated above to ensure our experiments are the upmost refined and animal welfare costs are minimised.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3R guidance available from https://nc3rs.org.uk/3rs-resources

Procedure related guidance available from https://researchanimaltraining.com/

Laboratory Animal Science Association (LASA) guidelines: https://www.lasa.co.uk/current_publications/ FRAME experimental design workflows: https://frame.org.uk/resources/experimental-design/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly view the NC3Rs website (https://www.nc3rs.org.uk/) to stay on top of advancement in 3Rs, as well as attending seminars as appropriate. We are in regular discussions with NACWOs and NVS to ensure our experimental methods are the best possible.

187. Genes, diseases and lifestyle influences on the musculoskeletal 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
    - 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

Bones, Joints, Bone Cancer, Arthritis, Secondary Bone Cancer

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant
Rats	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to improve the understanding of how genes, diseases and lifestyle influences musculoskeletal health. The ultimate aim is to identify and develop new drug targets to maintain a healthy skeleton throughout the life course, and/or combat the detrimental physiological changes that occur in 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?

Musculoskeletal disease is the second greatest cause of disability world-wide, affecting over 1.7 billion people, and having the fourth greatest impact on overall health. In the UK, one in two women and one in five men over the age of 50 will break a bone because of osteoporosis and 1/3 of older fracture patients die within 6 months of hospital admission. Osteoarthritis (OA) has undergone a substantial increase in incidence, with the prevalent disease burden of hip OA alone meaning that between 30 and 40 million European citizens suffering pain and disability. In the context of cancer, once a primary cancer (i.e. breast or prostate) metastasises to bone it becomes incurable, whilst the brutal treatment and poor survival statistics for primary bone cancer has not change in the past 40 years. These staggering facts makes it important for us to undertake this work to try and find new treatments and cures for Musculoskeletal diseases.

What outputs do you think you will see at the end of this project?

Outputs will include new information that will advance the scientific understanding of how our genes and our lifestyle affect musculoskeletal health and development of diseases that effect the skeleton. We are hoping to generate new information on how and why musculoskeletal diseases occur - in particular we are focused on disease such as osteoporosis, osteo- and rheumatoid arthritis, primary bone cancers such as osteosarcoma, Ewing Sarcoma and Chrondrosarcoma and secondary cancers that also affect the skeleton such as secondary breast and prostate cancer. This new information could include; identifying how our diet or our activity levels/exercise affect these diseases; how genes affect the ability of diseases to develop; how proteins in our bodies might also affect our skeleton and disease development. We will share this new information via scientific publications and press releases, and to also generate other outputs such as patents for new drugs.

Who or what will benefit from these outputs, and how?

In the short term the academic, medical and pharmaceutical community will benefit from this work with the publication of new knowledge. In the longer term this will translate to benefit for patients and the wider public (due to the socio-economic impact of reducing disease burden on society) as new and better treatments are developed. This has the potential for considerable impact as musculoskeletal disease which are the second greatest cause of disability world-wide, affecting over 1.7 billion people, and having the fourth greatest impact on overall health.

How will you look to maximise the outputs of this work?

We already collaborate with other academics nationally and internationally and we will seek to increase our network of collaborators during the course of this project. We will also seek to establish collaborations with the pharma industry and clinicians to help translate the outputs of this work. We will regularly disseminate new knowledge via presentations and scientific and patient focused conferences as well as publishing our work in scientific journals and in the popular media via press releases blog/website articles (both TUoS and those of our funders).

Species and numbers of animals expected to be used

- Mice: 7500

- Rats: 1100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice and rats in this project as they are the smallest mammals with musculoskeletal systems and genes similar to humans. The models we are proposing to use are already established and considerably refined. Rodents also have the added advantage of being amenable to genetic manipulation allowing the functional characterisation of specific gene products.

Typically, what will be done to an animal used in your project?

Animals may undergo injection of substances, surgical procedures, injection and establishment of tumours, alteration in diet and bone loading (either passive or via increased exercise i.e. treadmill running).

The duration of the experiment will not exceed that required to obtain meaningful results (can vary from one week to establish tumour take to several months due to slow growing tumours ie chondrosarcoma).

Number of procedures: mice 5200, rats 1100

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects for the animals during the project include those associated with anaesthesiology and surgery (i.e. pain, infection, wound dehiscence) which will be brief/for the duration of that step in the procedure. Mechanical loading/implants may impact the animals due to mild lameness which may persist for the duration of the procedure (2weeks+) but that does not impede the animal’s ability to eat and drink. Repeated anaesthesia and recovery periods may result in weight loss due to preventing normal feeding. Development of tumours in the animals may cause low level pain or again mild lameness if located on the hindlegs and which may persist for the duration of the procedure (2weeks+) but that does not impede the animal’s ability to eat and drink. Alterations to diet to specifically mimic a nutritional deficiency may result in a significant and easily detectable disturbance of an animal's normal state. The presence of tumours may also induce weight loss.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severity will not exceed moderate in the vast majority of cases/procedures in each category of animal.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Modelling the complex interaction between bone and or tumour cells and the environments in which they grow in patients as well as the effect of lifestyle choices (i.e. diet and exercise) is simply not possible in cell culture. The interventions and procedures needed to understand these interactions cannot be used in patients themselves. Where possible when the effects of agents on single populations of cells (bone or tumour cells) are evaluated, this will be done in vitro.

Which non-animal alternatives did you consider for use in this project?

We have considered and do use in vitro cell culture and modelling to predict interactions and outcomes in this project. We are constantly developing better in vitro models (ie multi-cell line cultures, organoids and we will use these as much as possible to ensure that we only use animal models when absolutely necessary for the project in question.

Zebrafish have been used by many researchers as an alternative to mouse studies, and we have considered this and where appropriate we would collaborate with colleagues who are experts in this field.

Why were they not suitable?

The alternatives are at the single cell (or at best tri-cell) level and do not fully recapitulate the intact animal and the complex interactions that takes place locally and systemically, nor do they consider the endocrine and paracrine influence therefore minimizing their clinical relevance.

Zebrafish are evolutionarily more distant from humans than mice and have some significant differences that mean they are not suitable for the majority of the aims of our project. Zebrafish bones only show few bones with trabeculae, long bones are absent and whilst Zebrafish do have growth plates, only a small portion of chondrocytes become hypertrophic - this makes them unsuitable for studying primary bone cancer as it predominantly occurs in long bones and especially during growth spurts involving the growth plate and chondrocyte hypertophy. Zebrafish bone marrow is fatty and does not harbour a site for haematopoiesis - again this make them an unsuitable model for looking at bone cancer metastasis as we, and others, believe and have demonstrated that cancer cells may hijack the haematopoietic niche to enable metastatic spread and as part of the seeding of distant organs (pre-metastatic niche).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 use over a 5-year period and predicted use in the coming 5 years taking into account planned research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The systems we are using have been used in previous studies and we have done statistical calculations to define the minimum numbers of animals required to obtain significant data. All experiments are designed using the NC3Rs Experimental Design Assistant a nd or an individual study protocol to fully consider the numbers of animals needed. Where possible we will also use repeated in vivo measurements (i.e. in vivo CT, BLI of tumour burden) over time where possible to reduce the number of animals needed to obtain statistically meaningful results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use pilot studies were necessary to optimise the number of animals we need where we do not already have this information. We also have an aim to use computer modelling to help us to reduce the use and number of animals in our studies. In addition, we will share samples with other projects –this is common practice within our laboratory/institution.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice and rats in our project and well-established experiment models for the diseases we are researching. The main animal models we are using have all been previously validated and published before, and we have refined the protocols over many years of experience, and we are using protocols that cause the least pain suffering and distress.

Why can’t you use animals that are less sentient?

Mice and rats as they are the lowest order of mammal that is still physiologically, anatomically and genetically similar to the human. As we are investigating lifestyle influences (ie diet and exercise) we can't use animals that have been terminally anaesthetised. Most of the disease we are researching also occur later in life (with the exception of primary bone cancer although this is still post-natal) and so we cannot use animals at a more immature life stage.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The main animal models we are using have all been previously validated and published before, and we have refined the protocols over many years of experience. The welfare of the mice and rats will be strictly monitored (i.e. enhanced monitoring post-operatively, daily welfare monitoring and the use of the mouse grimace scale for assessing pain). Appropriate pain relief during our protocols will be achieved through appropriate levels of analgesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the LASA "Guiding Principles for Preparing for and Undertaking Aseptic Surgery". Procedures for establishment and monitoring of tumour growth, metastasis and animal welfare we will follow best practice as outlined in "Guidelines for the welfare and use of animals in cancer research" Workman et al., British Journal of Cancer, 2010.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly receive updates from our NC3Rs Regional Programme Manager and we will attend local, national and international workshops and conferences; and read current peer-reviewed literature to stay informed of new advances. Our Named Veterinary Surgeon also provides regular updates to project holders on best practice and advances in the 3RS. We will actively engage in implementing these advances in our practices during the project.

188. Immune cross-talk between the oral and distant mucosal barriers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Periodontitis, Immune response, Mucosal Immuntiy, Inflammation, T cells

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's the aim of this project?

The aim of this project is to determine immune mechanisms that drive oral inflammation and better understand how oral inflammation contributes to the pathogenesis of inflammation at distinct mucosal barriers. Mucosal barriers are sites that separate the internal body from external environment and include sites such as the gastrointestinal tract, lung and skin.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Periodontitis, is commonly referred to as "gum disease" and the most severe form of oral inflammation. It is the most common chronic inflammatory disorder of mankind; affecting

almost 50% of the global population. Moreover, it has been predicted that almost 80% of the global population exhibit less severe forms of oral inflammation, such as gingivitis. Not only is oral inflammation therefore an incredibly prevalent pathology, but periodontitis has been associated with many other systemic diseases. For example, individuals with periodontitis have been shown to exhibit increased severity and/or incidence of rheumatoid arthritis, cardiovascular diseases, Alzheimer's Disease, diabetes, chronic obstructive pulmonary disease (COPD) and inflammatory bowel diseases (IBD). Thus, better delineating the immune mechanisms that contribute to the maintenance of oral health would not only promote the development of better therapies for periodontitis, but may also have implications for the treatment of other inflammatory conditions.

This work investigates the immune mechanisms which reinforce health within the oral cavity, defining how they become un-controlled during oral inflammation. Moreover, our work will outline how immune dysfunction within the oral cavity and initiation of periodontitis can contribute to the development or exacerbation of inflammation at distal mucosal barriers.

What outputs do you think you will see at the end of this project?

Two key outputs will emerge from this project that will advance our knowledge of oral inflammation and its systemic consequences. First, information generated from these studies will contribute to our understanding of how immune responses are appropriately controlled at the mucosal barriers of the mouth, yielding key information on important pathways that can go wrong during the development of oral inflammation, and more specifically periodontitis. Secondly these studies will provide some of the first mechanistic insights into the immune-based communication between the oral and distant mucosal barriers, as our approaches will outline how oral inflammation can contribute to extra-oral inflammatory diseases such as inflammatory bowel disease (IBD). We predict that the data generated within these studies will have a positive impact on the treatment of both periodontitis and as well as other diseases such as IBD, providing not only novel treatment options but also suggest mechanisms to stratify patients or identify patients at risk of developing both of these two, hugely impacting disease.

To maximise these benefits data generated from the studies undertaken will be effectively disseminated to the research community, (including academic and industrial researchers, medical and dental clinicians). This will be achieved through; i) publication of data in academic journals; ii) presentation of data at seminars and conferences and iii) through public engagement events. In this way data generated within this project will rapidly inform the work of others and promote scientific discovery within our research community.

There is a key secondary benefit that will also arise from undertaking this work; our work will advance research methods, through refinement and/or enhancement of current methods as well as through the possible development of new transgenic lines.

Who or what will benefit from these outputs, and how?

Scientific Community: This programme of work will generate new data, research tools and advance research methods which will be shared with the scientific community with the ultimate effect of promoting scientific discovery. This impact will occur in both the short term - following publication or talks but, overall support scientific advancement worldwide in the long term. Data generate will benefit researchers who focus on immunology and oral and gastrointestinal inflammatory diseases. However, our work will be more broadly applicable to other fields including microbiology, development and systems biology.

Healthcare Sector: Outputs from this research will, in the longer-term, have the potential to impact the healthcare sector, both medical and dental clinicians. This work will provide basic biological insights into the drivers of periodontitis and the mechanisms by which this disease impacts distant mucosal barriers, such as the gut or lung. It is hoped our studies will provide novel therapeutic targets for the treatment of oral, gut and lung pathologies targeted at the very accessible oral cavity. We also hypothesize that our studies could support the development of better mechanisms of patient stratification.

Biomedical Industry: Data generated from this project will also support the biomedical industry, as findings from our research are translated into the clinic. This impact will occur in the longer-term as novel therapeutics and/or treatment strategies emerge from our data sets.

General Public: The importance of oral health is often overlooked in the face of pathologies which drive more considerable suffering or lead more obviously to shorten life-spans or life-quality.
Information obtained from this project can be used to engage the general in the vital importance of oral health, highlighting the impact poor oral health, and subsequent development of oral inflammation, could have on completely distal organs.

How will you look to maximise the outputs of this work?

Dissemination of new knowledge: Output from this work will be published in highly visible (high- impact) journals which are read by a wide audience. Following publication of data in scientific papers we will make use of social media platforms to further publicise our work, including contacting daily newspapers and magazines (which have run stories on our work in the past). Alongside this, participants undertaking work on this project will be supported to discuss the work widely, presenting seminars and giving talks both locally and globally.

Collaborations: We already have in place a global network of academic and clinical collaborators that we will engage with as we develop this work programme. Outputs with collaborators will be maximised by regular meetings and data exchanges.

Publication of Negative Data: Although not common we have a strong track-record of publishing negative results. We will continue this whilst undertaking this project, ensuring publication in the most appropriate journals possible.

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.

The studies outlined will utilise juvenile and adult mice, which may be genetically modified. These life stages are employed to examine mice with teeth and/or mice just proir to tooth eruption. Mice are the most appropriate species for these studies as the systems which we

are studying here (the immune system at the oral and distal mucosal barriers such as the gastrointestinal tract and lung) are well reproduced between mice and humans.

Typically, what will be done to an animal used in your project?

Approximately 70% of the animals utilised on the project will undergo a procedure to investigate oral inflammation and the impacts oral inflammation has on immune health at distal mucosal barriers. To achieve this the following approaches will be used:

Oral inflammation will be induced in mice. This will be via one of three methods, each representing a key tenant of the pathology of oral inflammation. Either (1) periodontitis will be induced by the placement of a silk ligature around the second molar tooth. (2) The oral barrier will be damaged by abrasion with a sterile cotton applicator. Or (3) periodontitis and oral barrier damage will both be induced. For all these procedures mice will be anaesthetised and oral inflammation will result.

Altering the immune response at distal mucosal barriers. Here we will assess the impact of oral inflammation of immune function at distal mucosal barriers, such as the lung and gastrointestinal (GI) tract. We will initially explore impacts of periodontitis on gastrointestinal immunity. To investigate alterations in gastrointestinal immune responses one of three approaches will be used, each will allow us to examine the impact of oral inflammation on a distinct aspect of GI immune responsiveness.

Prior to, and/or, during these procedures the immune system may be manipulated, through application of reagents to target specific cell types (e.g. through use of antibodies or tracking agents) or through transfer of cell populations.

In addition to these procedures, mice will be used in this project for breeding, and for provision of cells and tissues for ex vivo studies

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may experience mild adverse effects such as temporary stress or brief pain and discomfort. Temporary stress may be induced by a brief period of restraint, for example in order to administer an injection. Mice may experience a brief period of pain and discomfort following induction of oral inflammation, through any of the three methods outlined, as well as following blood sampling. In these instances the stress and discomfort will be transient, specifically ranging from a few minutes to less than 12 hours.

Weight loss will occur after the following procedures (1) ligature-induced periodontitis, (2) administration of antibiotics, (3) generation of chimeras and (4) gastrointestinal bacterial challenge. In all cases this will typically be in the range of around 10%, with this weight loss being transient, for example ranging from a) following ligature placement weight is usually recovered within 48hours to b) following chimera generation weight is usually regained by 3 weeks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The studies outlined in this project will result in the following proportions of animals actually experiencing the following severity ratings:

Sub-threshold – approximately 20% Mild – approximately 30% Moderate – approximately 50%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no good in vitro or in silico models able to reconstitute the complex interactions occurring between oral tissue, host immune cells and microbiota and extra-oral mucosal barriers. The aim of my research is understand how immune responses are appropriately controlled at the oral barrier, and how immune dysregulation at the oral barrier can impact immune responses at distal sites; in vivo models provide a unique window to probe this.

The mouse is a good model to understand the human oral barrier, as the immunological network and tissue structure is similar in mice and man and the pathophysiology of oral inflammation similar in mouse and man.

Inbred mouse strains will be used, allowing uniformity between animals and experimental repeats.

There is considerable use of knockout and transgenic animals, which are not available for other species. Transgenic mice are already generated, are well characterized and available for use to assess gene and cellular functions.

Which non-animal alternatives did you consider for use in this project?

In vitro: Approached that include cell-lines or co-culture systems (where multiple cell types are cultured together). We utilise such approaches as these to i) inform the animal studies to be undertaken and ii) further validate targets and pathways identified in animal models.

Human samples: we have an active research program obtaining and examining gingival samples from human dental clinical cohorts. With detailed clinical information and patient meta-data such samples can be used to correlate immune phenotype with disease characteristics.

Neither of these approaches can replicate the complex environment of the oral barrier or model the distal interactions with the gastrointestinal tract and therefore cannot replace the use of animals.

Why were they not suitable?

Although the above approaches are valid platforms for research investigation neither fully replace animal models for the following reasons: i) Our studies in human can only be correlative and will not mechanistically link cause and effect, which can only be achieved in animal models. ii) our research questions require complex in vivo systems to precisely define the complex interactions between heterogeneous cell populations in their tissue microenvironment and also how they may impact responses at sites distal to the initial challenge. To date these cannot be accurately modelled 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?

Our group has extensive experience with the methodologies and approaches outlined here and of running projects of a similar scope. Consequently, estimates of animal numbers are based firstly on previous experience with the models to be utilised and the types of data generated, and secondly with careful consideration of the experimental design.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Throughout the duration of this licence, we will continue to consider the design of each experiment (as is required as a PPL holder). As we generate new data sets these will be used to further refine our experimental design.

For this application we have utilised data sets already generated from experiments which have led us to undertake this project. These data sets, along with our research objectives, have been discussed with the institute statistician, allowing us to get specialist advice on the types of experiments that will be undertaken and the nature of the datasets that will be collected. This has supported development of appropriately designed experiments which will yield biologically relevant and interpretable data with the fewest numbers of animals possible. We will continue to liase with this expert as our project develops and new data is generated.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Throughout this project, optimal animal usage will be achieved by the following means:

Utilising the most effective and efficient means to breed transgenic mouse lines. This will be achieved by consulting local and commercial experts In addition we employ an electronic stock management tool to monitor our colonies allowing real-time checking and maintenance. In this way we will minimise numbers of animals bred whilst still achieving allowing us to undertake experiments from which we can generate relaible data.

Pilot studies will be employed to optimise experimental conditions when we are developing new approaches.

We will maximise the amount of data that can be gathered from a single animal by using the latest technologies and most efficient tissue processing methodologies.
We will archive all tissue possible from each experiment. We also make our banked tissue available to our collaborators.

We will discuss our findings and share data generated with collaborators and the scientific community (for example through depositing large datasets in online data repositories). In this way we will maximise the scientific knowledge than can be obtained from our animal studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project involves the use of models of mucosal barrier inflammation. In both cases we will employ the most refined models available to address our experimental objectives. For example;

For the establishment of periodontitis in mice we will use the ligature-induced model of disease. This model results in 100% disease incidence and the disease is also temporally identically in each mouse; meaning treatments and experimental manipulations can occur concurrently. This model is also widely employed with others in the research community and has the additional advantage that disease development can be halted – by simply removing the ligature. Finally this disease occurs over an acute time frame, meaning period of time for which the mouse is showing signs of inflammation will be kept to the minimum possible to address the research question.

For the gastrointestinal (GI) challenges, the models to be used have been selected to drive well- defined immune responses in the gastrointestinal tract, leading to acute inflammation which will resolve, i.e. none of the methods employed lead to the establishment of chronic inflammatory reactions and as such, mouse suffering is kept to a minimum. All GI challenges employed have been well optimised in terms of doing route, dosage and time post-administration in order to induce a robust and consistent response whilst causing the least harm and suffering to experimental animals.
Where we seek to administer reagents to experimental animals we utilise the most refined route of administration possible. Furthermore we always seek to minimise the numbers of doses of a treatment in order to achieve our objective, this may involve pilot studies to identify an optimal dosing regimen.

Why can’t you use animals that are less sentient?

We cannot replace these studies in mice with studies in less sentient species, such as insects or fish, to achieve our objectives. Such species lack a defined oral barrier which is

comparable to humans. Moreover, they lack the complex immune found in higher order species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Appropriate animal handling techniques will be employed and updates, which further reduce stress and discomfort, employed as they are developed.

After procedures animals will be closely monitored in line with the refinement controls listed with each experimental step, for example this will involve detailed monitoring of weight and condition. Where transient weight loss is expected following some procedures, soft food/mash will be provided on the cage floor.

All animals will have environmental enrichment in cages and whenever possible, not singly housed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will base our approaches on the best published practises, for example those endorsed by the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Myself, as well as those employed by me and working under this license, will stay informed on recent advances in 3R approaches by staying up to date with NC3Rs recommendations and developments. This information from the NC3Rs is obtained through interaction with their website, local seminars, reading their updates and published bulletins and also through liaising with their local representative. We also continue to discuss further refinement opportunities with the NVS and NACWO and through interaction with collaborators and the wider scientific community at conferences, workshops and seminars.

189. Investigating the role of Seminal Fluid proteins in male fertility in poultry

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

Seminal Fluid, Reproductive ageing, Proteomics, Strategic ejaculate allocation, Poultry

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	adult
Red junglefowl (Gallus gallus)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify proteins in the male seminal fluid that are associated with high fertility in populations of poultry.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Identifying proteins in the male semen that convey high fertility is important to understand how reproductive traits evolve, and can drastically increase the productivity of commercial flocks and enable more effective husbandry.

What outputs do you think you will see at the end of this project?

The project will generate a better understanding of fertility in poultry by identifying how the seminal fluid protein composition changes within and across individual male fowl, and how such changes are associated with male fertility.

Specifically, expected outputs will include new information on the following:

Compositional changes in the junglefowl Seminal Fluid (SF) proteome associated with sperm fertilising efficiency, caused by rapid responses to socio-sexual conditions (female availability, female sexual novelty, male social status).

Compositional changes in the junglefowl SF proteome associated with sperm fertilising efficiency, caused by reproductive ageing.

The causal effect of junglefowl SF variation on sperm fertilising efficiency.

Characterisation of the SF proteome of Domestic fowl (commercial broiler breeder) males and patterns of variation across and within individual males.

This new information will be disseminated through scientific publications and communication to stakeholders and the lay public.

Who or what will benefit from these outputs, and how?

Understanding how seminal fluid proteins affect male fertility in poultry will benefit directly the Poultry Industry. The outputs of this project will inform strategies to better manage fertility and productivity of commercial flocks. These outputs will also be relevant to the management of animal welfare because Increased fertility will reduce the frequency of matings and hen exposure to males.

More broadly, the outputs expected of this project will collectively inform the following fields:

Andrology, assisted conception, sperm cryopreservation. Advances in the study of fertility and reproductive physiology in a vertebrate model may have relevance for the study of human fertility and especially research into declining sperm quality.

Biosecurity and Food security. Many SF Proteins in fowl and other taxa have antimicrobial properties. The reproductive tract hosts a diverse microbial community, including potentially zoonotic or pathogenic species. While little is known about sexual transmission in poultry, male semen has a diverse microbiota. The analysis of the SF proteome and seminal microbiome can therefore have major implications for the monitoring and management of livestock biosecurity and health.

Reproductive Physiology. The project represents one the most comprehensive studies of SF Proteins in birds, providing novel information on fundamental reproductive processes such as sperm maturation and metabolism, and the biological roles of seminal fluid.

Behavioural Ecology and Evolutionary Biology. Pioneering work on fruit fly accessory gland proteins have generated burgeoning interest in the role that SF Proteins play in postcopulatory sexual selection, sexual conflict and intersexual coevolution and reproductive barriers. The present project will establish an avian model system, informing

new avenues of theoretical and empirical investigations (male strategies of ejaculate allocation and phylogenetic analyses of SFP evolution rates).

Conservation Biology: The identification of avian candidate SF Proteins in fertilising efficiency will potentially benefit research into captive reproduction of endangered species, e.g. by informing more effective protocols of artificial insemination, the screening of individuals for selection in breeding stocks and sperm banks.

Understanding and exploiting Functional Genomics. The project will generate a wealth of new information on whole genome proteomics of a vertebrate model system. This will play a significant role in furthering our understanding of functional genomics and genome evolution, by contributing to genome annotation, identifying candidate genes and informing gene networks underpinning fundamental biological functions. These advances may in turn aid in the development of new genomic tools.

Ageing and Lifelong health. The project will directly benefit research into senescence, ageing and lifelong health, by providing a systematic characterisation of age-dependent compositional changes in the SF proteome and associated consequences for age-related changes in fertilising efficiency.

Cell, Cancer Biology. The study of cells secreting SF Proteins helps understand cell-cell communication, signalling pathways, and important infections and diseases: Drosophila accessory glands are used to study human prostate cancer. Nanotechnologies: SF often contains nanovesicles involved in molecular trafficking. We have found evidence that vesicles in junglefowl SF may be involved in delivering scaffolding molecules to maturing sperm. The study of these vesicles can inspire nanotechnologies, e.g. nanomedicine.

Bioinformatics. We will generate bioinformatic resources and help develop novel analytical tools of these data.

How will you look to maximise the outputs of this work?

Through the publication of scientific results, sharing of datasets, presentation of key findings at conferences, collaborations with other researchers and with industrial partners (e.g. commercial poultry breeders), stakeholder workshops and through a dedicated website.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 220
- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This research project seeks to establish the role of seminal fluid proteins in fertility in poultry flocks with a view to inform future strategies to improve fertility in commercial flocks. The project therefore necessarily focuses on two animal system: (a) a population of

red junglefowl, and (b) broiler breeder flocks of a widely used commercial line. The red junglefowl is the wild ancestor of the domestic chicken and as such represents a fundamental reference point against which to compare domestic chickens (e.g. to identify genes that may have been lost through domestication and artificial selection). Broiler breeders represent that parental stocks of broiler meat chickens. Broiler lines, which have been artificially selected for meat production tend to display suboptimal fertility rates and are therefore the most salient focus of this research project. Because the project focuses on seminal fluid proteins transferred during mating, it could not be conducted without studying sentient, sexually mature individual red junglefowl and broiler breeder that display their natural reproductive behaviours.

Typically, what will be done to an animal used in your project?

Individual males will be exposed to up to 40 semen sampling sessions per year, but typically 15 sessions per year, with at least 48 hours rest between each collection.

Females will be used to in staged mating to obtain semen samples from males, each female will be exposed to typically 15 and up to a maximum of 30 trials/year interspersed by a minimum interval of 2 days. Some females will be artificially inseminated (AI) with standardized amounts of semen, each female will be exposed to typically 4, and up to a maximum of 7 AI trials every year typically interspersed by a minimum of2 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect this work to have adverse effects or negative impacts on the welfare of the birds involved in this study.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Red junglefowl - 100% Mild. Domestic fowl - 100% Mild.

What will happen to animals at the end of this project?

- Kept alive
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project studies the seminal fluid proteins that male fowl release during mating. Thus, the project requires live, sentient, sexually mature birds.

Which non-animal alternatives did you consider for use in this project?

We have considered *in vitro* approaches, and we have implemented these whenever possible. These approaches involve studying the interactions between sperm cells and seminal fluid in the lab.

Why were they not suitable?

in vitro approaches are suitable to experimentally investigate the causal role of seminal fluid proteins on sperm fertilising efficiency (e.g. swimming velocity). However, their use is limited. First, *in vitro* approaches still require obtaining semen samples from sentient birds. Second, *in vitro* approaches cannot replace sentient animals for aspects of the project, which focus on mating behaviour and related behavioural responses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals needed for this project have been estimated based on effect sizes from previous experiments and our 20-year experience. Patterns of red junglefowl sperm and seminal fluid allocation behaviours have been well characterised by our research group in the recent past. In contrast, almost nothing is known about sperm and seminal fluid allocation in natural matings in commercial domestic chicken populations, including broiler breeders. The project will use a well- characterised study population of red junglefowl to study patterns of variation in seminal fluid proteins across matings under a range of socio-sexual conditions, following well-established experimental protocols used by our research group on this population. This part of the work will generate exhaustive information on how sperm fertilising efficiency is affected by changes (including age-dependent changes) in certain seminal fluid proteins. We will then screen a smaller number of broiler breeder males to compare and contrast the data from red junglefowl with seminal fluid compositional patterns and their relationship with sperm fertilising efficiency in this commercial line. Specifically, the junglefowl work will identify seminal fluid proteins related to fertility and we will investigate patterns of variation in these proteins in the broiler breeders. Because of this design, the project will seek to use more junglefowl than broiler breeders.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will take advantage of professional statisticians as well using our own experience. The number of animals used has been reduced by refining experimental design to follow ARRIVE criteria as closely as possible, e.g. through blinding, randomisation, and use of adequate controls including paired designs. Specifically, the experimental approach will utilise individual males as their own control through within- male comparisons following exposure of a bird to different experimental conditions in a randomised order. This design is powerful in that it minimises the noise caused by inter-individual variation. The species of animals investigated through mild experiments can be used on numerous occasions without any increase in harms to them. Estimates of the number of animals needed for

these experiments reflect an effort to minimise the number of animals while ensuring sufficient statistical power.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will make extensive use of the information gathered from substantial pilot studies and preliminary work. Detailed information on individual birds will enable us to select the most appropriate birds for each experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project will study red junglefowl and domestic chickens (broiler breeders), these two organisms represent the most salient comparison to investigate variation in male fertility in poultry. The project investigates the seminal fluid proteins that males release during mating, and thus the project cannot be conducted without the use of live, sentient, sexually mature animals. We will use the most refined procedures available to minimise pain, suffering, distress or lasting harm. Semen will be collected through non-invasive techniques, which our research group has refined over the past 20 years. These techniques represent the method for collecting natural semen samples that is most refined and causing the least amount of pain, suffering distress or lasting harm in these animals. The project will also use artificial insemination, a routine poultry husbandry practice, to study the fertilising performance of individual semen samples. This technique has also been used by our research group with no observed adverse effects, and represents the method to study fertilising performance in vivo that is most refined and minimises pain, suffering distress or lasting harm.

Why can’t you use animals that are less sentient?

The project investigates the role of seminal fluid proteins in released by males during mating, and as such it must study sentient sexually mature individuals. Given that its goal is ultimately to improve the management of fertility in poultry flocks, the project can only use galliform species (e.g. Gallus sp.). The use of less sentient animals such as insects (e.g. Drosophila) or even passerine birds would not generate relevant results because of fundamental differences in the reproductive biology across these taxa (e.g. male accessory glands, sperm physiology, ejaculate size, female sperm storage organs, seminal fluid proteome...).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures utilised have been refined by our group over the last 20 years, and we will incorporate any new additional refinement as and when it becomes available. All birds

utilised in this project will be acclimatised to being comfortable in the presence of humans and being handled. We will increase post- procedure monitoring compared to pre-procedure monitoring in both protocols. As per standard policy of the Establishment for the integration of new species, housing and husbandry of the Broiler breeders will be subjected to increased surveillance by the NVS in the initial stage of the project.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For experimental design we will follow ARRIVE guidelines. https://arriveguidelines.org

Specifically, for poultry we will consult the following resources:

https://www.gov.uk/government/publications/poultry-on-farm-welfare/broiler-meat-chickens-welfare- recommendations

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/3192 92/Farm_Animal_Welfare_in_Great_Britain_-_Past_Present_and_Future.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Mostly through the University 3Rs Newsletter. In addition, the PI has been Chair of the Departmental Animal Welfare Ethical Review Board (AWERB) and member of the University Animal Care and Ethical Review Committee (ACER) for several years. Both AWERB and ACER review developments in the 3Rs at different scales (from within the University to international) at termly meetings. As such, the PI is kept abreast of advances in the 3Rs on a termly basis, and attends 3Rs day events regularly organised by the University.

190. Preservation of Pig Liver and Kidney for Transplantation, Using a Novel Solution

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
- 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

Transplantation, Preservation, Kidney, Liver, Novel Solution

Animal types	Life stages
Pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine whether LS-A preserves abdominal organs better than in use UW solution, in static cold storage preservation, using a large animal model (the pig).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Organ transplantation not only saves lives but enhances the quality of the life of recipients allowing them to live long healthy and fulfilled lives. This costs society and in particular the NHS far less than having a critically ill person who takes up hospital time, treatment (such as dialysis prior to kidney transplant) and money; indeed many patients return to full time

careers after successful transplantation so pay tax and put money back into the country. Children who have organ transplants return to school and many go on to further education, have lifelong careers, get married and have children of their own. One thing that is clear is that the better the organ preservation solution the higher the chances of the organ working and the patient surviving.

There are currently around 6,500 people on the UK transplant waiting list and for the last year that there is data for, nearly 500 people died whilst waiting for a transplant (NHS Blood and Transplant data 2017), and in the USA about 20% of people will die waiting for a transplant. Transplants often do not go ahead due to the organ being not suitable and decisions about their use having to be taken quickly, largely due to limitations related to the length of time that organs can be safely stored before transplantation. This is because the length of time an organ can be out of the body prior to being transplanted is dependent on the effectiveness of solution which has been used to perfuse and preserve the organ. For example, at present the maximum storage time for liver using the current "gold standard", UW solution, is less than about 12 hours and for kidney only up to about 24 hours, even when the organs are considered to be in ideal condition before retrieval.

LS-A has been developed by a group of researchers for use in abdominal organ preservation and experimental data has shown that LS-A can extend the static cold storage period successfully for organs for liver and kidney transplantation.

Older, or less fit donors who are referred to as extended criteria or marginal donors are being used much more frequently now, as due to better medical and surgical care, people are surviving longer and not dying after such things as road accidents and brain haemorrhages. It is believed that using machine perfusion is one way to enable organs from these types of donors to be checked in a more effective way to see if they are suitable for transplantation and gives time to manipulate the organ and or the recipient if needed.

In order to combat the general shortage of organs available for life-saving transplants, older, or less fit donors who are referred to as extended criteria or marginal donors are being used more, including organs retrieved from DCD (donation after cardiac death) donors, and donors with fatty liver disease for example. These "extended criteria donors" are being used much more frequently now and it is clear that they need improved methods of organ storage to ensure function after transplantation. One method is machine perfusion after initial static cold storage, in particular a method known as Hypothermic Oxygenated PErfusion (HOPE). It is believed that using machine perfusion is one way to enable organs from these types of donors to be checked in a more effective way to see if they are suitable for transplantation and gives time to manipulate the organ and or the recipient if needed. Currently UW- MP (UW for machine perfusion), or an identical formulation sold under different trade names, is the only clinically available solution for HOPE. Paradoxically, one major failing of both UW and UW-MP is that they contain a high concentration of hydroxy ethyl starch (HES), which is known to be highly toxic. LS- A does not contain any HES.

Currently there is a need to optimise not only the solution used for static cold storage but also for HOPE as in clinical practice UW needs to be used for the initial flush and cold storage period and then exchanged for UW-MP for HOPE. A single improved formulation able to work effectively in both situations is needed. LS-A may be this solution so it needs to be tested for HOPE after static cold storage using both ideal and marginal organs.

What outputs do you think you will see at the end of this project?

At the end of this work, we should be in a position to do a clinical trial, using LS-A for the preservation of kidneys and livers to keep them in a better condition than the preservation

solutions on the market at the moment can do. It will also allow organs to be kept for longer periods than is possible at present, and this in turn will give time for the manipulation of factors in both the organ and the recipient, which will mean that the organs will have a better chance of lasting in the recipient for longer. Organs from extended criteria donors will be kept in a better condition and will be able to be manipulated so that the recipient will recover quicker and have a better quality of life for a longer period.

Who or what will benefit from these outputs, and how?

Long term the patients who will benefit will be patients in chronic organ failure waiting for a kidney and/or liver transplant. By being able to preserve organs for longer and having time to manipulate the organ or the patient will mean there are more organs of better quality for transplantation, so hopefully more patients will get transplants which will last longer. This in turn will be better for the health service, as the patient will require less care, and society in general as many of the recipients will be able to return to work, have less time off work so help the economy of the country.

Medics and scientists will also benefit as it will allow time for the organs or patients to be treated in different ways so that the transplants will be successful. Many of these treatments have yet to be discovered but it is believed that stimulation of certain cells and suppression of others may help in this process.

How will you look to maximise the outputs of this work?

It is intended that all the data from this work will be published in peer reviewed journals and presented at conference both nationally and internationally to disseminate the results.

Once clinical trials start, it is expected that other groups around the country and abroad will also be involved in these trials so as the largest cohort of patients and organs will be clinically tested.

Species and numbers of animals expected to be used

- Pigs: 12

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult female Landrace-Great White Hybrids Pig are used as these are the type which are used at our University, so no new breeds will need to be introduced. Adult pigs are used as they are the nearest in size to those found in human organ donors and will weigh about 30 – 40 kg.
Pigs are used in this experiment because the anatomy of the internal organs of the pig is similar to that of a human, making the surgery and procedure as near to what would be seen in human transplantation as is possible.

Typically, what will be done to an animal used in your project?

This process will kill the pig while still under anaesthesia. We plan to harvest kidneys and liver. Organs will be kept cool and will be flushed with a appropriate solution to keep them in optimum condition for storage and later in vitro studies. Each pig will be sedated All surgical procedures on pigs under this project license will be carried out while animal will be terminally anaesthetised. Each pig will be sedated and subsequently anaesthetised by a competent personal licence holder with expertise in pig anaesthesia. A human surgeon with a personal licence to carry out regulated procedures on pigs will then perform surgical procedures that include laparotomy, cannulation of peripheral and deep blood vessels, perfusion of organs with preservatives collection of large blood sample for later use, and removal of organs for subsequent in vitro studies on harvested organs.

What are the expected impacts and/or adverse effects for the animals during your project?

The pig will be brought to from the establishment approximately 18 -24 hours before the induction of anaesthesia to enable it to acclimatise to new surroundings. It will be sedated prior to surgery and anaesthetised so should suffer no abnormal behaviour or tension.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals will be killed during the operation. The pigs will be sedated and all procedures will be carried out under anaesthesia from which the pigs will not recover.

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 show that any organ preservation has been successful whole organs need to be used as when an organ transplant takes place the majority of the time it is a full organ which is used, the only exception being when lobes of liver or lung are used on tiny children. The only way that this can be done is by using whole animals. Looking and testing the effect of the solution on the whole organ rather than just certain cells is required.

Earlier work was done on rats but pigs are to be used in this research as pig kidney is multilobular and multipapillar, with a complex network of blood vessels, also found in humans but not in mouse or rats, so they are the closest there is to the human model.

Which non-animal alternatives did you consider for use in this project?

Some of the elements of the solutions have been tested initially on cell lines prior to their addition to the solution. If there were adverse events seen with the cell lines then these

were not added to the solution and were replaced without testing on whole organs or animals. We have now come to the stage when we need to use whole organs.

Why were they not suitable?

After preservation of the organs they will be reperfused with blood to mimic what happens following organ transplantation. Blood used in the reperfusion so that different biochemical perimeters specific to the organ transplanted can be tested after different lengths of time to ascertain how well the organs are functioning, for which we require the full organ. For liver we will be also collecting bile and for the kidney we will be collecting urine both of which require the full organ as more than one cell type is involved in their production.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 preliminary studies to test viability and/or difference between two preservative solution being widely used in the preservation of organ before transplantation. We think 12 animals will give us statically viable information to see the differences in two solutions.

Many different types of tests on the organs, tissue and blood means plenty of results can be obtained despite the low number of animals used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals used several strategies have been used. Firstly, as much as possible of each animal will be used, so both the liver and kidneys will be retrieved. Secondly blood will be taken for use in reperfusion parts of the experiments rather than being bought in which would entail more animals being killed. Thirdly each time when a pig is going to be used, other researchers who can use other parts and tissues will be informed so that they can take what they can use.

If a pig dies unexpectedly, the organs will still be taken and used, as this situation mimics another way in which organs are retrieved for transplantation in humans, called extended criteria donors. This means that if other researchers are euthanizing a pig for any reason, we may be able to retrieve and use the organs, again soon after death.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Rather than transplant the organs into other animals, to reduce the number of animals used it is intended that we use an isolated perfusion rig, to mimic transplantation. This equipment allows us to reperfuse both kidneys and livers at the same time, outside of the pig, so each pig will have the liver, both kidneys and blood removed from it. The rest of the

pig will be offered to other groups to see if further use can be made of it.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 type of animal used will be Landrace-Great White Hybrids as these were used in earlier in the development of the solution and are the breed of pig which is kept at the University farm; therefore no new species will be introduced and there will be the minimal of movement of the animals which will cause them less stress.

Pigs are social animals and should not be kept alone for prolonged periods of time, therefore the pigs will be brought from the establishment where they are kept to where the surgery will take place, only 24 hours prior to it being required for surgery. This gives it time to acclimatize after the move but not long enough for itto get lonely.

All work on the pig will be done under anaesthetic so it will not feel any pain and will be euthanized whilst still under anaesthetic. All work on the pig will be done under anaesthetic so it will not feel any pain and will be euthanized whilst still under anaesthetic. Anaesthesia on the pig will be carried out by a competent personal licence holder with expertise in pig anaesthesia. Surgical procedures will be carried out under aseptic conditions by a competent transplant surgeon with a personal licence to work on pigs. No animal will recover from anaesthesia. This will mean that the pig will be caused the least pain and suffering possible for these procedures.

Why can’t you use animals that are less sentient?

Earlier work was done on rats but pigs are to be used in this research as pig kidney is anatomically more complex, with a lobular structure and a complex network of blood vessels, which are similar to those found in humans but not in mouse or rats, so they are the closest there is to the human model.

The pig liver is closer to human liver anatomy than rat or mouse liver and it is widely accepted that the porcine hepatocyte is the best non-human model to use as an alternative to human hepatocytes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Each pig will be brought to the University only 24 hours in advance of their use. Where the pigs are reared they are in a group as they are not solitary animals. They will only be at the site for a short period and will be sedated prior to anaesthetic so that it will not suffer many adverse effects. The pigs will be monitored by the University Veterinary Surgeon at all time from the point of sedation, through the general anaesthetic and surgery until the point

where it is killed. The monitoring will be the same as would be done during a routine operation on an animal, and if at anytime the pig is thought to be suffering initially we will try to alleviate it but if this is not possible it will be killed by a humane method so that it does not suffer any longer.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animals (Scientific Procedures) Act 1986 (ASPA)

EU Directive 2010/663/EU

UK Research Integrity Office Code of Practice guidelines. ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

A computer search will be carried out prior to each period of work with the pigs to see if there are any changes or advances in the 3Rs which need to be accounted for with the work. This will include checking on the Home Office site, NC3Rs site and also the Royal Veterinary site We will also check with the University Veterinary Surgeon to see if they have any knowledge of any changes which would impact on this work.

I already receive updates from NC3R and any changes regarding the use of the animals will be done in accordance to their advice.

191. Isolated tissues for the study of cardiac ischemia and cardiovascular 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

cardiovascular disease, myocardial infarction, ischaemic heart disease, arrhythmias, heart failure

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	neonate, adult
Guinea pigs	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of these studies is to understand how the heart works and, more importantly, how it goes wrong. Our ultimate aim is to improve the diagnosis and treatment of heart disease. We will examine the influence of pharmacological, dietary or physical treatments on the cellular processes involved in cardiac injury.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular disease remains the number one cause of death in Western countries accounting for over 30% of all deaths (Virani et al., 2021). It is predicted that cardiovascular disease is also set to become the leading cause of death in developing countries. This rise in cardiovascular disease reflects a significant change in dietary habits, physical activity levels, and tobacco consumption worldwide as a result of industrialisation, urbanisation, economic development and food market globalisation. High blood pressure, high blood cholesterol, obesity and chronic type 2 diabetes are among the major biological risk factors.

Virani SS et al (2021) Heart disease and stroke statistics - 2021 Update. *Circulation* 143 (8): doi.org/10.1161/CIR.0000000000000950

What outputs do you think you will see at the end of this project?

The principle outputs will be publications and conference presentations.

Who or what will benefit from these outputs, and how?

In the short term we expect the scientific community to be the primary beneficiary. However, it is the longer term aim of this work that either directly, or through the work of others, new products, drugs or life-style changes will be developed to substantially prevent, or improve the treatment of, cardiovascular disease.

How will you look to maximise the outputs of this work?

At all times work in our laboratories aims to be open and collaborative. We have many active collaborations around the UK and the world (i.e. USA, Israel, Germany, Canada etc). We will maximise our outputs be ensuring publication in international high-profile journals.

While positive data is sadly always easier to publish, we believe that scientifically reliable negative data is as important. A recent example of this relates to the mechanism of action of the SGLT2 inhibitor class of drugs in the heart. Based on a small number of flawed studies, the idea that SGLT2i's exert their effects by inhibiting the cardiac Na/H exchanger has gained traction. We have recently published negative data showing that this is not the case. See doi: 10.1093/cvr/cvaa323 and doi: 10.1093/cvr/cvab184.

Species and numbers of animals expected to be used

- Mice: 4500
- Rats: 1500
- Guinea pigs: 450
- Rabbits: 450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

All studies will be confined to either mice, rats, rabbits or guinea pigs. No experiments are proposed that involve *in vivo* ischaemia and reperfusion and/or the recovery from

anaesthesia. Over the past 30 years we have continually refined our in vitro techniques such that we (i) limit all studies within our laboratory to small animals (mice, rats, rabbits and guinea pigs), (ii) all of our studies ultimately involve terminal anaesthesia and/or *in vitro* experimentation, with the exception of the mild pre-treatment protocol (Protocol 2) and the breeding and re-derivation of transgenic mice, and (iii) collect tissue for molecular/biochemical studies from the same animals or hearts as those used for the assessment of *in vitro* function, where possible.

A significant proportion of these studies will be carried out using adult mice. The mouse has become a pivotal experimental system for animal-based cardiovascular research, due to the rapidly increasing availability of genetically modified models of various cardiovascular diseases. The availability of such mice has undoubtedly had an enormous impact on the understanding of cardiovascular disease. Transgenic or mutagenic mice will be created, either on other Project Licences or in other laboratories elsewhere in the world, and maintained under authority for use on this Project Licence. Provision is therefore made for the breeding of transgenic or mutagenic animals on this project licence. Specific provision is also made for re-deriving mouse lines (Protocols 5-7) if necessary and for cryopreservation of fertilised eggs.

In addition, studies will be carried out using the adult rat. This animal has been well characterised and its heart and tissues are robust and suitable for isolated studies. However, a number of important physiological differences exist between mice and rats and other species including man, such as altered action potential characteristics, subtle changes in calcium handling, the extent of myocardial collateral blood flow, etc. These differences are useful in some experiments as they allow specific cellular systems and characteristics to be investigated, however, it is essential that specific studies are also performed in species in which particular cellular systems are more closely analogous to the human heart. Such species include the guinea pig and rabbit (where cellular physiology and calcium handling are more similar to humans). Some studies will therefore be conducted in these species.

In a very limited number of studies, tissues will be isolated from neonatal animals after a terminal procedure. Such studies are necessary to (a) provide data on the neonatal heart and its maturation relevant, for example, to neonatal cardiac surgery, and/or (b) to provide isolated cardiomyocytes that can be readily cultured and transfected (Note: adult cardiomyocytes dedifferentiate in culture).

Typically, what will be done to an animal used in your project?

Most of our studies under this license will be performed i*n vitro.* The licensed procedures described here will mostly be limited to the terminal anaesthesia of healthy animals for the excision of tissues (usually the heart) which will be subsequently studied *in vitro.* For a typical experiment, animals will be anaesthetised and, after thoracotomy, the hearts and associated major vessels will be excised [Protocol 1]. This terminal anaesthesia would involve a single procedure of an anaesthetic agent delivered via an appropriate route according to species. Typically the duration of such procedures would be less than 5 mins.

In some experiments, animals may be pre-treated prior to terminal anaesthesia with drugs or dietary manipulations. The duration of such pharmacological pre-treatments will not exceed 28 days while dietary manipulation may be initiated from weaning.

What are the expected impacts and/or adverse effects for the animals during your project?

Terminal anaesthesia and heart excision: The vast majority of experiments will only involve terminal anaesthesia and therefore there are no chronic adverse effects to consider.

A small number of studies, *in vivo* pharmacological pre-treatment up to a maximum duration of 28 days will be conducted to examine the effects on heart function after excision from the animal. Some pharmacological interventions capable of modifying the myocardial response to ischaemia or reperfusion in vitro require administration prior to the excision of the heart. It is therefore proposed that a number of animals will be pre-treated with such agents,administered via appropriate intravenous, intraperitoneal, intramuscular, subcutaneous or oral routes at appropriate concentrations according to manufacturers instruction and/or known data. As some of our work involves the development and characterisation of novel therapeutic approaches, it is impossible to anticipate specific details of the exact substances, concentrations or routes of administration.

However, it is essential to stress that in ALL CASES, animals pre-treated in this manner will be monitored to ensure that, in itself, such treatment DOES NOT EXCEED THE MILD SEVERITY LIMIT designated in each protocol. Hence, administration will initially involve low doses that will only be increased to the required dose once detailed monitoring has been conducted to ensure no adverse effects are induced. In some studies, drug pre-treatment may be combined with dietary manipulation as described. A small number of animals pre-treated with either drugs, dietary manipulation or both may be subjected tocardiovascular monitoring by non-invasive imaging (e.g. echocardiography) or implantation of telemetry probes.

Dietary manipulation: Prior to tissue excision, some animals may be fed a modified diet. Such diets will be formulated to modulate the levels of existing nutrients (without causing imbalance that will result in overt and immediate adverse health effects). Examples of such dietary manipulation may include adding dietary achievable levels of fish-oils, fat-feeding, cholesterol enriched diets, lowering the amount of iron, etc. Such diets will only modify the levels of nutrients that occur naturally in a Western human diet. This modification will not limit calorie intake, or render the animals malnourished, induce excessive weight loss or any of the classical signs of listlessness or overt ill health.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All species - Mild (95%)

All species - Moderate (5%) (Telemetry)

What will happen to animals at the end of this project?

- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Ischaemia is a complex multifactorial pathology that cannot be replicated in cell culture models. Although it can useful to study individual signalling pathways in *in vitro* cell cultures, the contribution of these pathways to ischaemia/reperfusion injury can only be usefully assessed in whole hearts or freshly isolated tissues. Isolated cardiac cells are required for the detailed investigation of cellular mechanisms. There are no immortalised ventricular myocyte cell lines available, which makes it necessary to isolate cells from neonatal or adult animal hearts. We have attempted to replace animals (i) by the increased use, where appropriate, of cell culture systems (such as human iPSC-derived cardiomyocytes, HEK293 and A10 vascular smooth muscle cells) to probe specific cellular mechanisms, and (ii) by the use of human tissue derived from routine operations to replace some animal experiments. However, human ventricular tissue is not readily available, and is almost invariably from diseased explanted hearts and cannot typically be vascularly perfused.

Which non-animal alternatives did you consider for use in this project?

Cell culture

Human Cardiac Induced Pluripotent Stem Cells (hIPSC) Computer modelling

Why were they not suitable?

Cell culture: Ischaemia is a complex multifactorial pathology that cannot be replicated in cell culture models. Although it can useful to study individual signalling pathways in in vitro cell cultures, the contribution of these pathways to ischaemia/reperfusion injury can only be usefully assessed in whole hearts or freshly isolated tissues. While hypoxia (with added potassium elevation and acidosis) recapitulates some features of ischaemia, it falls far short of being a suitable model of the multi-cell- type, 3-dimensional paracrine organ that is cardiac tissue. The 3-dimensional structure is also a prerequisite to study complex cardiac arrhythmias which cannot be mimicked in a dish.

hIPSCs: Recently experiments on cardiac hIPSC cells (Human Induced Pluripotent Stem Cells) have become popular. While these cells recapitulate some features of adult cardiomyocyte they are notoriously variable in phenotype, immature and a poor model for may physiological and pharmacological studies (Funakoshi and Yoshida, 2021).

Computer models: While computer models are useful in confirming and validating our understanding of physiological processes, they can only be parameterised and used in parallel with in vivo and in vitro models. They are an addition to, but not a replacement for, the use of real biological tissues.

Funakoshi S, Yoshida Y (2021) Recent progress of iPSC technology in cardiac diseases. Arch Toxicol. 95(12):3633-3650. doi: 10.1007/s00204-021-03172-3.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The large number of animals required (in particular the large number of adult mice) reflects the large number of licensees (~10) working on this project and our experience over many years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible, all experiments are blinded and randomised. In biology, measurements made are often complex and variable. Therefore, in order to be able to compare between protocols, all experiments need to be repeated a number of times, averages taken and appropriate statistics applied. By rigorously controlling the conditions of our experiments, this variability can be minimised. However, for most measurements we need to repeat experiments at least 6 times. For some less predictable variables (such as arrhythmias) this number may increase to 10 or more. Each experiment may also require a number of different groups (different drug concentrations, control groups, different genetic backgrounds etc). This gives typically approximately 30-60 animals per experiment (this includes some that fail to meet the experimental inclusion criteria standard, for whatever reason). Each experiment may take 3-4 months to conduct; hence, each licencee may require 2-300 animals per annum. Most licencees will use predominantly mice or rats in their experiments. Animals will also be required for the production of myocytes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The numbers of breeding pairs will be minimised to the number required to just meet our requirements. Unfortunately, due to variations in ischaemic tolerance across the oestrus cycle in rats and mice, most experiments will be conducted on male animals. This therefore requires culling of female mice before weaning.

Where possible tissue will be shared. Heart homogenates are frozen and a tissue bank managed locally to provide tissue across a number of investigators. On a routine basis, myocyte isolations are shared across a number of investigators. Typically, one adult rat heart can provide sufficient myocytes for up to eight 6 well tissue culture plates, or around 50 samples for biochemical analysis. Similarly mouse myocytes can be shared across projects. The number of animals used will be kept to a minimum by distributing cells from each preparation between several users.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority (90%) of the studies described in this license involve terminal anaesthesia only. Pain, suffering and distress will therefore be minimal. There is clearly always some stress in the induction phase of any anaesthesia but all PIL holders working under this license will be appropriately trained to minimise this distress.

In the small number of animals where drug pre-treatment is involved prior to terminal anesthesia (10%), all PIL holders working under this license will be appropriately trained and supervised. In this case there will be regular interaction and guidance from BSU staff and the NVS. Again, in the majority of cases, in this small cohort of animals, pre-treatment with drugs will be achieved using the least stressful route of administration - this is often by inclusion of a palatable drug in drinking water or diet.

In an even smaller small cohort of animals (<5%), drug pre-treated animals (and placebo controls) may be non-invasively scanned while under sedation or anaesthesia to measure cardiovascular function using modalities including ultrasound (echocardiography) or MR Imaging; OR, some animals may be monitored using implanted telemetry devices to measure physiological variables such as blood pressure, heart rate, ECG etc. These invasive measures will be used rarely and, when used, appropriate analgesia will be given.

Why can’t you use animals that are less sentient?

The majority (90%) of studies involve the isolation of tissues from insentient unconscious animals under terminal anaesthesia. All experiments where animals are pre-treated with drugs or dietary manipulation will be performed only on animals post-weaning. While less sentient animals such a zebra fish etc have a huge contribution to make to the understanding the cardiovascular system, they are better suited to developmental studies. Ischaemic heart disease, heart failure and arrhythmias are largely diseases of the adult human heart. It is unclear whether less sentient animals either suffer from heart disease or indeed make appropriate models for the human in this regard. Hence, the mammalian heart is used for the majority of such studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As the majority (90%) of the studies described here only involve terminal anaesthesia and tissue isolation, there are no issues about post-operative care. In the small number of pre-treatment or pre- termination monitoring (echo, telemetry etc) we will liaise with the NVS to ensure all procedures meet with evolving best practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Home Office and institutional protocols for induction of anaesthesia, sedation, and animal care. These include the LASA guidelines on aseptic surgery. ARRIVE guidelines will be followed in study design and in all publications depending on editorial constraints.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All researchers involved are constantly reading the scientific literature and continually refining our experimental approach. We also have regular updates from BSU staff and the NVS and institution-led training on animal welfare and the 3Rs.

192. Development of Therapeutic Agents to Treat CNS Disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neurodegeneration, Psychiatry, Treatment, Therapeutic Efficacy, CNS disorders

Animal types	Life stages
Mice	adult
Rats	adult
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to provide a service to support discovery and development programmes for therapeutic agents for Central Nervous System (CNS) 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 is currently a huge unmet medical need for effective therapeutic agents to treat psychiatric disorders as well as for the symptomatic and disease modifying treatment of neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease.

What outputs do you think you will see at the end of this project?

Compounds, drug candidates or drugs that have efficacy in animal models of CNS disorders that could progress to clinical development.

Publications in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

The ultimate benefit will be patients living with psychiatric diseases (for example depression and schizophrenia) or neurodegenerative disorders (for example Alzheimer's disease and Parkinson's disease) for which there is no medicine to slow or halt disease progression. In the short-term scientific knowledge may highlight disease related mechanisms relevant to their aetiology that would then direct research and testing new therapies in disease relevant models.

How will you look to maximise the outputs of this work?

Where possible - and if needed, with agreement of clients - data will be published in peer reviewed journals and presented at appropriate scientific meetings.

Species and numbers of animals expected to be used

- Mice: 1600
- Rats: 2550
- Guinea pigs: 700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rats and mice are the predominant species used in preclinical research into CNS disorders as they have a fully developed central nervous system, are the most common species type used in preclinical CNS research for which there is a wealth of scientific literature and experimental expertise. Moreover, the CNS circuitry and systemic physiology that determines the pharmacokinetic profile within the CNS and the associated pharmacodynamic response that underpin therapeutic efficacy is comparable to humans.

Typically, what will be done to an animal used in your project?

Animals used in the project may be naïve, genetically modified (e.g. expressing a disease relevant phenotype), undergone surgical procedures to lesion specific neuronal processes or been subject to pharmacological manipulation.
Naïve or genetically modified animals may undergo surgical procedures to implant devices to allow assessment of cellular or neuronal function in the CNS or for determining levels of test compound following acute or chronic treatment. Separately, animals which express pathologies or symptoms associated with a CNS disorder (e.g. using genetically modified animals, or animals with discrete lesions to particular neuronal systems) may be tested to

measure CNS relevant behaviours (e.g. related to cognition, anxiety, motor function). In these studies, test compounds will be dosed acutely by systemic or direct injection, but on occasions animals may be dosed chronically to assess the effect of more prolonged treatment to determine if pharmacodynamic effects are maintained, or if a test agent has neuroprotective properties.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals undergoing surgical procedures for implantation of cannulae, microdialysis probes, sensors or catheters recover within a few hours from anaesthesia and a full recovery from the procedure within one to two days. Following stereotaxic delivery of a neurotoxin adverse effects may include neurological impairment (e.g. motor dysfunction following a dopaminergic lesions) that may last for the duration of the experiment (typically no more than 60 days).

Most of the animals are not expected to show signs of adverse effects that impact materially on their general well-being following test compound administration. No more than 5% of animals are expected to show clinical signs of a mild or moderate severity because of unpredicted side-effects of administered compounds (typical effects may include reduced appetite, weight loss, reduced activity, and diarrhoea).

Expected severity categories and the 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 that most animals will experience moderate severity due to the necessity to undergo surgical procedures and/or chronic administration

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no current *in vitro* alternatives which would model the complex *in vivo* processes involved in absorption, distribution, metabolism and excretion or safety of candidate drug molecules. Similarly, there are no *in vitro* models for CNS conditions that usually involve integrated responses not only from the already extremely complex neuronal tissue, but also from the immune or the vascular system.
There are currently no alternatives to *in vivo* testing able to replicate the complexity of a whole organism accurately enough. As such *in vivo* studies remain essential in the search of novel therapeutic agents for CNS disorders.

Which non-animal alternatives did you consider for use in this project?

There are alternative strategies we can use, particularly for studies investigating a specific cellular response to target manipulation, where *in vitro* studies can be carried out on cultured or primary cells. We will also propose and use *ex vivo* experiments where possible, for instance, using synaptosome preparations to measure neurotransmitter release or use brain slices to measure electrophysiological responses of test compounds. We will also seek alternatives to *in vivo* testing by exploring the use 3D *in vitro* models, with particular interest on those able to replicate Blood Brain Barrier (BBB) properties.

Several *in vitro* and *in silico* assays have been developed to replace the use of animals for the initial steps of CNS drug discovery. These assays are designed to ensure that only compounds predicted to cross the BBB and with acceptable profile for efficacy, selectivity and suitable ADME (adsorption, distribution, metabolism & excretion), will be tested on live organisms. Although these experiments are usually performed by the client, we have in house capability to carry out such studies.

Why were they not suitable?

In all cases the scientific question to be answered in a proposed experiment will be carefully studied and *in vitro* and *ex vivo* alternatives will be considered. However, using animals *in vivo* offer more predictive methods to assess the complex integrated processes controlling the absorption, distribution and interaction of a test substance with a molecular target, and its functional effect on neuronal processes relevant to CNS disorders.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 typical study design and number of projects predicted over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals to be used is influenced by previous extensive experience in designing and running studies of this type.

The minimum number of animals used is also underpinned by an understanding of the variation between animals, the sensitivity and reproducibility of the methods being used, and with statistician advice being sought when needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies are frequently used when developing models and sharing of model information with collaborators 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The overall premise of the license is that the most refined, most relevant, and less invasive method will be used at each stage. We will expect the greatest number of compounds to be tested in models with minimum burden on the animals (duration of study, stimulus required for experimental window and endpoint). When the experimental question justifies additional burden to the animal (i.e. surgery, lesioning, chronic dosing) we will aim to keep such burden to minimum levels.

Surgical techniques and post op-recovery standards will be continuously monitored and revised. Surgical procedures, including preparation, surgery, and recovery care, will be performed in keeping with current best practice guidelines (LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

With chronic studies, the dose route will be carefully discussed with the client and alternatives to repeated injections such as implantation of mini pumps or subcutaneous pellets will be contemplated.

We will continuously monitor the literature and involve the Named Animal Care and Welfare Officer (NACWO) as much as possible to help us to implement the latest animal husbandry and environmental enrichment practices.

Substances administered to animals by injection or gavage will be in the smallest volume commensurate with the aims of the procedure. The administration of substances to animals by infusion will also be at the lowest flow rates and for the minimum period practicable. Substances will be delivered in non-toxic biocompatible vehicles. The route of administration will be carefully determined by a number of factors such as the solubility of the substance, PK properties, number of administrations over time, volume, vehicle used or projected dosing route in humans. We will determine the route of administration in compliance with LASA guidelines (Good Practice Guidelines - Administration of Substances) and relevant literature with regards to substance administration (i.e. Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider, Turner et al (2011)).

Why can’t you use animals that are less sentient?

Species of choice will be primarily rat or mouse, depending on the pharmacological properties of the therapeutic agent, endpoint employed and the knowledge of the target. Adult animals will be preferred as they have a more developed nervous system and less sentient species do not allow application of the same methodological approaches, or translational aspects for inferring a drug's therapeutic potential.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continually review our peri- and post- operative care procedures and each of the PPLs learn from each other with respect to procedures and refinements. Our NACWO/NVS/NIO attends regular national meetings and provides us with feedback on learnings.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experiments will be planned so they can be reported in accordance with the NC3Rs' ARRIVE guidelines and we will conduct regular literature reviews to ensure that our work aligns with current best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By regular discussion with the Named Information Officer (NIO), NACWO and the Named Veterinary Surgeon (NVS) in addition to receiving regular updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Scientific Research (NC3Rs). The 3Rs are also an item agenda during our quarterly Animal Welfare and Ethical Review Body (AWERB) meeting.

193. Mechanisms controlling anti-tumour responses in primary and metastatic disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cancer, Immune checkpoints, T cells, Therapies, Metastases

Animal types	Life stages
Mice	juvenile, neonate, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to determine how different cellular interactions control the anti-tumour immune response in primary and metastatic disease and how targeting certain mechanisms, termed immune checkpoints, alters this. Armed with this information we aim to determine how best to combine new treatments with the clinically approved immune checkpoint blockade therapies to further enhance the immune response in cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

One in two people will suffer from cancer during their lifetime. Successful treatment of cancer is hugely dependent upon the type of cancer and also how quickly it is diagnosed. Late diagnosis for many cancers results in at best a prognosis of only a few more years of life. Considering Cancer Research UK statistics (from 2010-2011) of the 21 most common cancers, 12 have ten-year survival of 50% or more, and four types – testicular cancer, malignant melanoma, prostate cancer and Hodgkin lymphoma – have survival in excess of 80%. Some cancer types, however, remain difficult to diagnose and/or treat, and ten-year

survival is less than 20% for stomach, brain, oesophageal, lung and pancreatic cancers, which actually has a ten-year survival of just 1%. In short cancer remains the single biggest health concern of humans, particularly in Developed countries where infectious diseases have been controlled through vaccination.
In the last 10 years, there has been enormous advances in exploiting immune responses to kill cancers. Striking successes include targeting immune checkpoints, which have revealed that even with late stage diagnosis, common cancers such as melanoma can be cured in some patients. CAR T cells have proven to be stunningly effective in curing some types of lymphoma. However, it remains the case that therapies that manipulate immune responses work for only some types of cancer and typically in less than 50% of patients with 'responsive' cancers. Furthermore, manipulation of the anti-cancer response through targeting immune checkpoints can come at severe costs in terms of adverse events and toxicity. The lack of a universal effect reflects an incomplete knowledge of how immune responses to cancer are generated and sustained in the face of multiple signals that limit these responses. In particular, metastatic disease remains far more resistant to immune therapies. Discovery research investigating the mechanisms that still limit the anti-tumour response, particularly in the context of immune checkpoint blockade can help establish combination therapies so that more cancer patients can benefit.

In this Project we will investigate how different immune cells interact to support the anti-tumour response in both primary cancer and in metastatic disease. We will focus on T cells as we know that these are the cells best equipped to kill cancer cells and able to respond to therapies such as immune checkpoint blockade. Using novel mouse models developed in our lab we are able to track cellular changes in real time. We can distinguish cells based upon how long they have been within the tumour and this provides critical new insight into understanding how mechanisms act to control the T cell response. We will model the effect of the licenced immune checkpoint therapies and determine exactly how these impact the T cell response to both primary and metastatic tumours. We will then investigate the mechanisms that restrain the response in the face of this therapy to identify potential ways to further enhance it. Finally we will then test these combinations of therapies seeking to identify those that significantly improve control of tumour growth, in both primary and metastatic disease. This research strategy can rationalise and inform how best to refine treatments designed to enhance anti- tumour responses.

What outputs do you think you will see at the end of this project?

The key outputs of this project will be:

a detailed understanding of what happens to the T cell response within primary and metastatic cancers and how targeting immune checkpoints changes this. This information will be published in scientific journals and potentially influence the design of therapies in cancer patients.

detailed characterisation of the how the other cells within tumours support the anti-tumour T cell response and how this cellular 'infrastructure' is altered by targeting immune checkpoints. This information will again be published in scientific journals and will support efforts to optimise therapy combinations.

testing of new therapeutic combinations, informed by our dynamic analyses. These will provide initial evidence supporting the combined targeting of specific mechanisms.
We have consistently published all our findings in high impact immunological journals and we will continue to do so as the best way of advancing understanding from our research.

In addition we will continue to present our research findings at scientific conferences, invited seminars, interactions with industrial partners and public engagement events.

Who or what will benefit from these outputs, and how?

Short-term benefits: Our detailed in vivo research will directly benefit researchers, particularly cancer immunologists, seeking to better understand the anti-tumour response and how this can be best enhanced. In addition to academic research, research teams within industry, e.g. pharmaceutical companies, can exploit our research findings to refine their efforts to manipulate key pathways operating in the cancer.

Medium and long-term benefits: Our research outputs and observations will feed through into both academic and industrial efforts to develop better combinations of therapies to enhance anti-tumour immunity. It will also benefit clinical oncologists considering treatment options for cancer patients and how different therapies might be combined for optimal effect. This will feed through into better therapeutic options for patients with cancers that show limited responses to current treatments and for which new treatment combinations need to be tried.

How will you look to maximise the outputs of this work?

Throughout my research career I have actively collaborated with many researcher in my field and shared expertise, research tools and models widely and prior to publication. I think this is an essential part of maximising the outputs from basic research. I will continue to do this and further continue to instil this philosophy in the researchers of my lab. Publishing our work in high impact journals with broad readerships provides an effective means of disseminating experimental data and findings. I am also very active in presenting research findings from my lab nationally and internationally through invited seminars and conferences. Finally, I have established collaborative interactions with pharmaceutical companies and will continue to develop these to maximise the influence of our research activity.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this project we will use mice to further our understanding of what happens to certain immune cells (called T cells) within cancers and why this happens. The environment within cancers has an enormous effect on the cells within these tissues. Furthermore, immune cells traffic between lymphoid tissues and the cancer. Collectively this is far too complex to accurately model through non-animal methods currently available (e.g. in vitro cultures). This really means that the only way to control for all these parameters is to study what T cells do within the cancers within a living animal. This ensures that the data we acquire is of maximum relevance to understanding the mechanisms that control the response in human cancer patients.

We have chosen mice as our animal study for the following three key reasons:
The main components of the mouse immune system shared by humans, making the mouse an excellent model of the human immune system.

The mouse immune system has been extensively studied already, meaning that there are an extensive range of reagents available to help careful study of what mouse immune cells do and why. This is important because it means we can use pre-existing data, we don't have to validate evidence in a new species and our research is not restricted by a lack of experimental tools.

There are many genetically modified mice available which enable detailed information about what cells do or how they behave. This is hugely important, particularly in this Project as it means we can understand how cells change over time and in specific environments, which cannot be captured by other approaches.

The immune system alters over the course of development, and our research is focussed on functionality in the adult. For this reason we will use adult mice.

Typically, what will be done to an animal used in your project?

In a typical experiment on this licence, tumour cells will be injected into a specific location in mice and these will grow into a cancer over the following weeks. These tumours will grow in sites where they cause very little, if any, discomfort to the mice. Most mice will additionally be injected (typically by intraperitoneal injection as a route that causes limited and transient discomfort) with reagents on multiple occasions over about 2 weeks. Typically this will be less than 6 injections.

In some experiments we will assess how cells are changing over time and in response to the injected reagents through labelling the tumour with light (using ‘photoconvertible’ genetically modified mice).The vast majority of experiments would last for approximately one month or less and at the end all animals will be humanely killed and tissues taken post-mortem for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

All mice on the project will experience temporary discomfort and stress from the anaesthetic event used for injection of tumour cells. In most cases one or two tumours will then develop at superficial locations (for example on the flank under the skin) where they cause very limited if any, adverse effects. Some mice will be given tumours in their colon, but this is done via an endoscopy and under anaesthetic and again these tumours cause very limited discomfort to the mice in most cases. In all these mice, the greatest impact will be the transient discomfort and intermittent stress of handling and injections. We will keep the number of injections to the minimum required and the typically the injection route will be intraperitoneally (an injection through the skin into the body cavity). Light exposure to temporally label tissue will be done under anaesthesia, thus limiting distress to the mice. The process of light exposure to tumours under the skin causes no adverse effects to the animal. Light exposure to colonic tumours is performed via a modified endoscope, again under anaethesia. In some experiments we will model lung metastases by injecting tumour cells intravenously which then accumulate in the lungs as a collection of very small tumours. These tumours, if left unchecked, can impair respiratory functions, but we will

ensure that mice with these lung tumours are killed at the earliest opportunity to study the tumours and certainly before respiratory function is noticeably impaired.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum expected severity on any protocol is moderate. This is because almost all mice on the licence will be given tumour cells that result in a local cancer or cancers growing in the mouse. These developing tumours cause minimal adverse effects to the mouse when carefully monitored and not allowed to grow very large. The greatest adverse effects experienced by the mice will be through multiple injections. The combination of tumours and multiple injections and photoconversion means that we cumulatively expect a moderate severity for approximately 80% of the mice on this licence. We expect that the remaining 20% will experience a mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To investigate what happens to the immune response within cancers and following treatment, it is impossible to properly assess this without studying a living animal and the different tissues and conditions that exist. The tumour environment is a hugely complex mixture of many cell types and these cells constantly interact and regulate what happens to each cell type. Furthermore, the majority of these cells are not static and both move between areas of the cancer and between the cancer and other tissues. In addition other parameters change regularly over time (e.g. nutrients). It is not possible to effectively model this environment nor the cells within it in a way that captures any of the normal cellular behaviours and interactions. Findings from only non-animal experiments would still then require extensive validation in an animal model before there relevance to human patients could be assessed and confirmed.

Which non-animal alternatives did you consider for use in this project?

We do exploit computer-based approaches to capture existing information from previous studies. With the advent of sophisticated cell sequencing and bioinformatics approaches, which we will continue to use throughout this project, computer-based studies form the major non-animal approach we use. These experiments can provide clear direction in which molecules to try to manipulate to control what happens to different immune cells.

We also considered organoids (cultures of certains cell types), which can enable the study of single cell types in a more 3D environment and even multiple cell types for example

intestinal epithelium organoids. We further investigated 3D culture approaches using a 'framework' approach where cellular interactions are enhanced.

Why were they not suitable?

We concluded that neither organoids nor 3D culture models would be appropriate as they cannot accurately model the cancer environment and this is key to controlling the immune response. Organoids cannot recapitulate the interactions of multiple cell types, which we know have huge influences on the immune response. Currently, no in vitro model (3D culture nor organoid) can recreate the complexity or dynamism of the tumour microenvironment nor appropriately model the entry and exit of immune cells.

We will look to develop patient tumour fragment analyses where by small pieces of human tumours are cultured for a few days and the effects of treatments can be tested. These experiments can help direct the in vivo research and ensure appropriate mechanisms are investigated.

Computer models are excellent at capturing descriptive data and we will continue to inform our in vivo projects where possible, but they do not provide the means to test 'cause and effect' nor determine functional outcomes. We will screen initial candidates using cell cultures in the lab and this will enable us to rule out some pathways/approaches that do not function as predicted.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 calculating the number of mice we will use, I have reviewed data over the last 10 years of my animal returns on my existing and previous PPLs. The number here reflects the need to maintain an extensive breeding colony to generate sufficient experimental mice across multiple genetically modified strains.
Furthermore, efforts to reduce experimental variability, for example through using age and sex- matched animals within an experiment, or the correct use of littermate controls in conditional knockout mice experiments, means that a large colony of mice for each strain is required to ensure that sufficient numbers of the desired mice are available. For individual experiments, the number of animals required is calculated based on data obtained from previous experiments, pilot studies, and information in the literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In designing this experimental body of work, we considered our previous experience of experimental models to investigate T cell responses and have also utilised the NC3R's Experimental Design Assistant. It is our extensive experience in using the approaches described here that really enables us to keep the number of mice used in this project to a minimum. We have selected only those experimental models that gave the most robust

and quantitative analysis of the intestinal T cell response and eliminated other experimental approaches where there was greater experimental variability or less clear means of definitively testing T cell function.

Through extensive research in the lab we have further optimised our understanding of the tumour models underpinning this project and identified the key time points that need to be assessed. This reduces the number of analysis points when mice might be killed. Through establishing and sustaining a productive collaboration with industrial partners, we are able to acquire reagents that have been extensively validated in vivo and generated to extremely high standards. This massively reduces the numbers of mice needed to test new compounds that we want to use in our studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We continue to monitor mouse usage on our projects by constant and careful consideration of the data and its appropriateness in addressing our experimental objectives. In developing our models we reassess group sizes as our experience develops and we will continuously look to use the minimal number of mice that provide robust experimental data adhering to standards of experimental reporting laid out in the Arrive 2.0 guidelines. In our publications we endeavour to fully report our data adhering to these guidelines to ensure experimental transparency and ensure that experiments do not need to be further repeated due to any ambiguities in their interpretation.

While our experimental models are all established and optimised, we will likely test new reagents in targeting pathways that might regulate T cell function. When this occurs, we will use small scale pilot experiments (n=3) to first test the novel reagent. Furthermore, through many years of active collaboration with industry, we typically acquire reagents from these collaborators (e.g. blocking antibodies for in vivo). This ensures numbers of mice used can be kept to a minimum as these items have been extensively validated previously and we adhere to the recommended dosing regimen in our pilot study, ensuring less animals need to be used in establishing the experimental setup in our models. Furthermore, the purity of these reagents is often substantially higher than what is provided through commercial sources ensuring less experimental variability and smaller group sizes.

Over many years of supervising my own mouse colonies, I have developed extensive experience in efficient breeding strategies. A key approach in our experimental models is that through tracking endogenous antigen-specific responses, the data is highly consistent between experiments (rather than in models where TCR transgenic T cells are transferred for example). This means that data from different experiments can be pooled to reach the desired number of mice per experimental group. Therefore we use mice as they become available, rather than trying to generate large cohorts and this is a considerably more efficient means of breeding the mice with less mice generated and not 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.

In all of our experiments we use mice to study the immune response to cancer and in most experiments we only use genetically modified mice. We have selected genetically modified mouse models that enable either dynamic labelling of tissues, to better inform of immune cell behaviour or models that provide further insight into specific mechanisms.

For example:

We use Photoconvertible mice as host for tumours and then violet light to label specific tissue (e.g. the tumour, the draining lymph node) to track cell migration in vivo and changes in cell phenotype and function in a given site. We have refined these approaches such that the adverse effects are kept to an absolute minimum (e.g. subcutaneous labelling rather than surgical exposure of the tissue to be labelled).

We utilise primary tumour models where the cancer grows either under the skin on the flank, in the mammary fat pad or in the wall of the colon. Here the tumour causes minimal adverse effects to the host and its growth can be easily monitored. This ensures that the tumours do not grow too large, which can potentially cause further adverse effects.

The tumour models used (mostly cell lines) express molecules that help refine our analysis and reduce experimental variation, thus maximising the detailed information generated.

We will use in vivo imaging (IVIS) of mice with tumours to ensure that we can monitor tumour growth. This is a non-invasive approach that can carefully monitor the size of cancers even when within tissues (e.g. lung, colon).

Why can’t you use animals that are less sentient?

Mice are the least sentient animal species we can use to study the anti-tumour response and inform human treatment as the cellular components of the immune system known to be key to the response to cancer, alongside tissues that control the response (lymph nodes) are present only in mammals (i.e. not, for example, fish). Our experiments further exploit the genetic modifications available in mice to maximise the detailed functional questions we can ask to provide critical new insight of relevance to human patients with cancer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have extensively refined our experimental approaches over the last PPL and continue to review our methodology. We have consistently worked with staff in the animal facility to refine experiments and will continue to seek their advice on this project. We have reduced the need for surgical interventions through developing ways that label cells without the need to expose the target tissue. Model refinement is an active and continual process that requires careful consideration of all stages of the experiments. This ethos is instilled within researchers in my lab.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All the researchers in my group are aware of the revised Arrive 2.0 Guidelines developed by the NC3Rs and recently published. We have consistently endeavoured to publish our research to the original ARRIVE standards and will continue to maintain this publication standard to ensure experimental transparency and reproducibility. In planning our experiments, we work to the PREPARE and LASA guidelines. This is taught to members of the lab when they arrive and actively discussed as researcher learn how to plan and then conduct in vivo experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Within our lab we continuously scrutinise the literature for experimental advances that might enable further refinement of our models, reduction in the number of mice used or ways to entirely replace in vivo experimentation. All members of the lab are actively encouraged to engage with this. We also engage with presentations and symposia organised by the animal facility and the NC3Rs and receive their emailed news updates. Both I and members of my lab have presented in 3Rs themed sessions, highlighting experimental refinements we have developed to try to benefit other research groups.

194. Understanding brain circuits in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neurodegeneration, Brain circuits, Epilepsy, Dementia, Drugs

Animal types	Life stages
Mice	adult, aged, juvenile, neonate, pregnant
Rats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to advance our understanding of how brain cells function normally and how their properties are changed in different neurological diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

Diseases of the brain including abnormalities in development (causing schizophrenia and autism) and also changes related to ageing (causing dementia) are still poorly understood. The research outlined in this proposal aims to further our understanding of both brain function during normal ageing, but also changes in brain activity in disease models. In particular diseases such as epilepsy, schizophrenia and different forms of dementia, are all related to changes in specific brain regions or brain cells. In order to find novel therapeutic treatments that could slow down, or even halt changes or loss of brain cells linked to these

conditions, we need a better understanding of the brain circuits involved.

What outputs do you think you will see at the end of this project?

The outputs of this work will include new information to share with the scientific community. The results from this work will be presented at meetings and also in peer reviewed journals.

Who or what will benefit from these outputs, and how?

Our results could inform the work of other scientists involved in research in the field and other related fields. Work published during the project could, therefore, have immediate benefit to the scientific community during the course of the project.

The results will also be of interest to pharmaceutical companies who are continuously developing novel approaches to treat different neurological conditions. In the group we work closely with pharmaceutical companies who will be interested in our results and data may inform the development of new projects which could be started during the course of this current licence.

In the longer term the results of this work will contribute to our growing understanding of the changes occurring in the brain in a range of diseases and will aid in the development of novel treatment approaches that could be moved into human clinical trials.

How will you look to maximise the outputs of this work?

I will publish the work conducted during this project in high impact journals; I engage with many collaborators both internally and in other Universities around the UK, Europe, USA, and Australia and have several joint publications with these collaborators; I have industrial links with large international pharmaceutical companies and have received funding both in the past and currently for our research. Some work may involve testing novel treatments that could ultimately move into clinical trials. Work from the lab will also be presented regularly at key scientific conferences both nationally and internationally.

I also have a long and successful track record in public engagement projects funded by Wellcome including art and theatre projects informing the public about schizophrenia and epilepsy which have reached large public audiences and resulted in appearances on BBC Radio 3 and Radio 4.

Species and numbers of animals expected to be used

- Mice: 6760
- Rats: 3760

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Adult rats and mice will be used in this project as these animals have been widely studied

and there is a large body of information concerning their anatomy and physiology that is already very well established. The brains of rats and mice contain all the same types of brain cells that are present in the human brain, and both rodent species generate patterns of brain activity in vivo that are similar to those observed in humans. The molecular mechanisms that contribute to brain communication are also very similar in all mammals.

The project will use rats and mice of all ages and both sexes. We are interested in studying changes in the brain in relation to both normal ageing, and also disease models of dementia such as Alzheimer's disease and Lewy body disease. We will therefore use rats and mice across the lifespan of the animals so we can follow both healthy ageing and disease progression.

Typically, what will be done to an animal used in your project?

The vast majority of the work on the project involves taking brain tissue for studies of brain cell activity in slices of brain tissue or for anatomical studies. For these experiments the animals are killed by terminal anaesthesia and the brain is then removed. It is possible to keep small sections of this brain tissue alive for a few hours and record activity of brain cells. In many cases no interventions will have occurred in the animals prior to tissue harvesting. Some animals may have undergone an intervention, e.g. a pharmacological treatment, given either by injection, or where possible in the drinking water or the animal may have undergone cognitive testing.

Some of the rodents used will have surgery for the implantation of brain recording devices. This is now a well-established technique and animals are then free to move freely in the home cages and eat and sleep while recordings of brain activity are conducted in awake animals. Such data gives us a true insight into normal brain activity and can be directly related to human EEG recordings. Implantation is done under general anaesthesia and there is close and careful monitoring (using score sheets) of the animals post-operatively to ensure there are no complications.

Some animals on this project will be used to study a neurological disease such as epilepsy and may, therefore, have had a drug injected into the brain to generate the abnormal activity associated with epilepsy. The injection is again done under general anaesthesia with close and careful monitoring of animals post-operatively. The techniques used have been developed so that animals typically have only very mild seizure activity, for example, twitching or freezing behaviour. We can then assess how epilepsy affects cognition and brain circuits and function and test novel therapies aimed at stopping seizure activity.

What are the expected impacts and/or adverse effects for the animals during your project?

Discomfort from the surgical implantation of the electrodes is expected immediately post-operatively but animals are administered pain relief for 1-2 days or as deemed necessary by veterinary advice.
Thereafter animals continue to thrive for months with the electrodes in place, just as in humans with deep brain stimulating electrodes implanted in the brain, who are able to continue with their lives.

Some animals in which seizures have been induced will have a change in behaviour during the seizures which can include facial twitching or freezing but in most cases these are transient (a few seconds) and not severe. Animals are monitored daily to ensure that

the seizures do not become too severe using a prepared score sheet. If seizures do become severe animals will be used immediately in other protocols or killed by Schedule 1.

Some of the transgenic mouse models of neurodegeneration that are used on the study develop Parkinson-like symptoms when very old e.g. tremor and rigidity, but animals in this project are usually used at an age before these symptoms occur. Again daily monitoring ensures the animals do not develop severe motor dysfunction symptoms.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

A lot of work is non-recovery ~50-60%.

Drug treatment protocols are moderate (~15%).

Surgical electrode implantation studies are moderate (~25%). Epilepsy models are moderate (10%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We cannot model the impact of novel treatments, for example, treatments to stop brain seizures in epilepsy without using animals. We need to assess the impact of interventions on cognitive function and network activity in the intact brain and this is only possible in either the whole animal of slices of living brain tissue. Some background studies can be performed in vitro in networks of living brain cells so reducing the number of animals needed for in vivo research.

Which non-animal alternatives did you consider for use in this project?

We work closely with computer modelling experts and modelling can help reduce the number of experiments but they cannot replace the use of live brain tissue.

In the lab we also use human tissue, taken from patients undergoing neurosurgical removal of brain tissue, for example, to gain access to a deep brain tumour. This data is highly valuable and allows us to validate our key findings from the rodents in human cortical tissue but it cannot replace all animal work.

Why were they not suitable?

Computer models rely on real data to generate the model so results from experiments are needed to input to the model. Computers do not work like the human brain as, obviously, they do not have brain cells nor any of the chemicals and molecules in the brain that effect function. We will not be able to understand how brain circuits function, for example, to consolidate memories using just a computer. However, we can test some predictions in a model and so reduce the number of experiments performed in animals.

The human samples we have access to are limited in number and come from patients who have severe disease (brain tumour) and who may have been on a range of medications. Furthermore, while very important this tissue is not normal control tissue.

Some manipulations such as controlling brain cells to stop epilepsy are in the early stages of development and need to be proven to work in animal models before any clinical trials in humans can be considered or would be approved.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experience and those used on the current licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3Rs Experimental Design Assistant. We perform pilot studies to test potential efficacy of a treatment or to obtain numbers for power calculations prior to a larger study.

Animals are used in a manner that maximises the data from one animal. For example, cognitive testing can be performed in an animal, then studies of brain cell activity and morphology can all be conducted using the same animal. Tissue can be obtained from multiple brain regions, and many different morphological studies can be conducted in a single animal, thus reducing the number of animals needed for any study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All transgenic animals from models of neurodegeneration bred in house are used for studies. We do not waste any animals. We use all male and female mice as comparing data from both sexes is important. In addition, heterozygous and homozygous animals are used for our main transgenic lines.

We collaborate and have published for over 20 years with network modelling researchers and we share our data with them. We will provide data to open access online resources which can then be shared by many other scientists in the field thus reducing the need for

others to conduct experiments.

We share other organs of the body (e.g. liver and heart) with collaborators both in internally and elsewhere in the UK and provide them with fresh or fixed tissue.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We obtain the maximum information from tissue that has been obtained from an animal killed with terminal anaesthesia. In addition, other experiments can be conducted under terminal anaesthesia where we can record brain activity for several hours. This greatly reduces the number of animals that are used for other awake behaving in vivo procedures. All the procedures in this project are classified as non-recovery, mild or moderate.

We have spent some considerable time working with the vets to develop surgical procedures that ensure minimal adverse effects. All surgeries are performed in the operating theatres under sterile conditions thus reducing the risk of post-operative infections. The surgery performed has been refined so that it can be performed quickly, reducing the time the animal spends under anaesthesia and thus speeding post-operative recovery. Design of the implants has meant minimal post-operative complications, such as failure for the wound to heal or the implant becoming detached.

The model of epilepsy chosen to use normally causes very modest seizure activity and most animals experience only mild symptoms of freezing or starring behaviour.

Why can’t you use animals that are less sentient?

We are studying brain activity in normal health and disease and, therefore, need to use an animal model that contains all the same brain cells as are present in humans. Rats and mice have the same types of brain regions, the same brain cells, and the chemistry and molecular properties of these brain cells are similar to humans. As outlined above we do as much of the research as possible using animals that have been terminally anaesthetised but if we want to understand, for example, how a treatment may stop an animal from having an epileptic seizure, we need to have an awake-behaving animal to assess this.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have, as outlined above, refined our surgery by developing small head implants that are fitted easily. Surgeries are all performed in the sterile operating theatres and we have check lists for daily monitoring of animal welfare post-operatively. We are in continuous discussion with the veterinary staff to consider ongoing refinements to the techniques

used.

For mouse breeding we try to use all mice, including heterozygous and wild type of both sexes so no animals are unnecessarily culled if possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow ARRIVE guidelines and continually look for ways to improve experiment reliability and thus reduce the numbers of animals we need to use. For example, we are looking at ways to randomise animal group selection and for experimenters to be blinded to the animal group. As discussed below it is not always practical for the experimenter to be blinded but where possible this will be implemented.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We continuously develop and refine our approaches throughout the duration of a study in discussion with the veterinary staff. There are seminars, regular leaflets etc. from our animal facility that also help inform us of new developments and the NC3Rs website.

195. Translational pharmacology for drug discovery

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- 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

Neurological, Central Nervous System, Pain, Inflammation, Translational

Animal types	Life stages
Mice	adult, neonate, embryo, 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's the aim of this project?

The aims of this project are to continue our efforts to facilitate and optimise the advancement of potential new medicines being developed by other drug discovery scientists (e.g. pharmaceutical & biotechnology companies, academic institutes) for neurological disorders, pain and inflammation. This project will provide evidence from pre-clinical rodent models of the likely therapeutic benefit in the clinic, including determination of the dosage required to produce such benefits and potentially the type of patient and clinical outcomes most suited to the new treatment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could

be short-term benefits within 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 societal burden of neurological disorders, chronic pain and inflammation are enormous. For example, the global opioid crisis resulted largely from the lack of appropriate treatments for chronic pain, schizophrenia is a lifelong condition with an onset in adolescence, and in the UK in 2018, dementia (including Alzheimer's disease) was the leading cause of death in women.

Pre-clinical data produced by this project will help identify the best new drugs/therapies for progression into human clinical trials, and potentially reduce the failure rate currently observed in the clinic.

What outputs do you think you will see at the end of this project?

The studies performed under this licence will contribute to the understanding of disease areas previously mentioned that have a clinical unmet need.

We aim to have a better understanding of the science in these disease areas, to develop new disease models/technologies that are highly translational to the human conditions. This will facilitate the development of new medicines/therapies that progress into human clinical trials, and benefit the patient population.

Where significant results are not subject to confidentially agreements they will be communicated more widely at scientific meetings or published (Publications/abstracts).

Who or what will benefit from these outputs, and how?

Throughout the duration of the licence, Short term - Pharmaceutical, Biotechnology companies and academic institutes will receive data allowing them to make decisions on progression of potential drug candidates/treatments to enhance the likelihood of success in the clinic.

Medium term - Will help Pharmaceutical, Biotechnology companies and academic institutes expand their knowledge around programs of work and potential candidates, including the effectiveness of potential medicines in disease area of interest, the mechanisms by which these treatments work, and potential adverse side-effects. This knowledge can be used focus research on the chemical structures most likely to benefit patients, and to halt animal testing of compounds which are unfit for progression. By halting the progression of compounds early will potentially lead to a reduction in the number of animals being used.

Long term - We aim to broaden the understanding of the mechanism of the disease modifying targets and aid the development of treatments that will improve the quality of life for the patients and carers.

How will you look to maximise the outputs of this work?
We will work both independently and with clients/collaborators to publish our findings/knowledge gain throughout the duration of the licence. We will disseminate findings by publications at industry conferences, via publications in the literature and via publication of online posters.

Species and numbers of animals expected to be used

- Mice: 36000
- Rats: 38000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Most of the animals used under this licence will be adult rats and mice (Including genetically altered animals), some animals may be juvenile, where they are required to be around 5-6 weeks of age, lower in weight range to animals over 7 weeks of age, where this will impact the study readout.

Genetically modified animals will be used because of human specific targets and disease specific mechanisms. Some genetically modified mice will be humanely killed as new-borns where tissues are required for cell culture, in order to be used for testing in non animal models and thus reducing the number of live animals used down the line.

Aged animals may also be used where we are investigating diseases strongly associated with the older patient population, such as cognitive/memory disorders.

Rats are widely used to provide data in drug discovery because there is a large body of data describing some of the similarities and differences between rat and human physiology.

Mice are used sometimes in situations where antibodies optimised for use in human, work via the target in the mouse but not in the rat. Also, because genetically altered animal models of human disease mechanisms are most commonly produced in mice.

Typically, what will be done to an animal used in your project?

Experiments/studies requiring surgery

Animals will be anesthetised and surgically prepared (area of incision cleaned/made sterile to reduce chances of infection) for the appropriate model such as by implanting electrodes/transmitters/transmitters onto specific brain regions to enable recordings of brain patterns or implanting a cannula into specific regions of the brain for administration of test substances, or a disturbance/trauma to a nerve for induced models for pain. Animals will be allowed sufficient time for recovery dependent on the surgical procedure performed. Animals will typically be treated with test treatments, either under the skin, into the abdomen or directly into the stomach and subjected to behavioural testing in response to a stimulus (such as mechanical or thermal), or to more natural behaviours (such as Burrowing or nest building).

Non surgical models/assays (Pain)

Animals will be assessed for behavioural response to a stimulus (such as mechanical or thermal), or to more naturalistic natural behaviours (such as Burrowing or nest building). Animals will typically receive some form of pain insult (either by an injection into the paw or sometimes the abdomen) causing a swollen or sensitive paw, they will then be treated with test treatments which maybe a single or repeated dose either under the skin, into the abdomen or directly into the stomach and re assessed for the behavioural measures.

Non surgical models/assays (Epilepsy)

Animals will typically be treated with test treatments which maybe a single or repeated dose either under the skin, into the abdomen or directly into the stomach. Animals will then be subject to either some form of electrical or chemical stimulus to cause seizure activity that is measured. Electrical stimulus is typically very brief (0.3-3 seconds) and the response/end point is very quick (10-20 seconds). Chemical stimulus is typically via single or repeated dose either under the skin, in to the abdomen and animals observed for a period of time, typically between 30 mins and 2 hours.

Non surgical models/assays (PK/PD or cognition)

Animals will typically be treated with test treatments which maybe a single or repeated dose either under the skin, into the abdomen or directly into the stomach. Animals will then be assessed for behavioural measures, either in a home cage or observation box for visual observations, or using an automated system designed to measure and record many behaviours such as general movement, grooming, rearing, circling, eating and drinking, behaviours may also be assessed using a rotating wheel, to look at effects of balance or sedation.

What are the expected impacts and/or adverse effects for the animals during your project?

Some level of pain is inevitable for evaluating test treatments in models of inflammatory and Neuropathic pain, but these are kept to a minimum and typically do not cause other changes in the welfare of the animals. For example food and water intake are normal, animals grow at the normal rate and the general health and behaviour of the animal is unaffected. Some animals show slight initial weight loss, but are provided with addition food sources and are monitored carefully.

Some animals may show changes in behaviour, following treatment with pharmacological agents to induce disease characteristics such as amphetamine induced activity, continuous stimulated movement.

Streptozotocin (STZ) induced neuropathy/nerve pain causes typical symptoms of diabetes, including increased thirst, increased urination, excessive sugar in the urine and high blood sugar levels. Therefore, animals will drink more than usual and this will be taken into consideration during the husbandry care. Post STZ injections, 2 % sucrose is added to the drinking water to avoid the initial drop in blood sugar levels, and animals will stay group housed to help maintain body temperatures.

With electroshock induced seizures, animals receive a single electrical current to the eyes or ears, to produce a clear seizure endpoint. After application of the current, animals may or may not achieve a generalised seizure, but are all killed within 10 seconds to minimise any suffering.

With chemical induced seizures, animals typically receive a single injection to induce seizure activity, depending on the disease model that it is mimicking, this may last for a short period of two minutes or for longer periods on 2-3 hours dependent on the model. Animals will be monitored at all times post dosing and are humanely killed when reaching the specified endpoints (seizure level) or at the end of the observation period.

With animals that have had electrodes surgically implanted into areas of the brain (EEG), some animals may have small swellings in the abdominal area post surgery, which typically show no sign of infection, discomfort or pain and normally resolves without intervention within 1 week.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 10% Sub threshold/non recovery
20% Mild
70% Moderate

Rat: 10% Sub threshold/non recovery
20% Mild
70% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Tissue or cell-based assays cannot replicate the impact of potential therapies in whole organisms, especially for the neurological conditions. For example, drugs destined for human treatment are usually given systemically (oral or by injection) rather than by direct application to the brain, and therefore they must survive digestive or metabolic processes and cross the blood brain barrier before having the desired effect.

The complexity of the mammalian brain cannot be overstated, and there are no computer simulations which even come close to mimicking that complexity, though they are useful for generating novel ways of thinking about problems in neuroscience. Human or animal tissue preparations can provide useful information at a molecular level (e.g. drug binding to a receptor of interest), and cell cultures can also be useful, but are also highly artificial, as the cells are grown outside their normal environment and without any connectivity with other parts of the brain. Whilst this information helps understand what a drug might do in an isolated preparation, research in a whole animal is still required.

No cell-based systems are in existence that can replicate the whole functioning organism.

A range of chronic diseases/disorders are covered by this licence, including cognitive deficits associated with Alzheimer's Disease, a range of pain and inflammatory conditions (Osteoarthritis/Rheumatoid arthritis), Neuropathic conditions (nerve damage, diabetes and chemotherapy induced neuropathy) and various forms of epilepsy. These are all conditions where the whole organism (i.e. intact nervous system) is required in order to measure a cognitive/painful/seizure response.

Which non-animal alternatives did you consider for use in this project?

The overarching aim of this project is, specifically, to provide animal research services for studies where this is most appropriate. For this reason, consideration of non-animal alternatives is beyond our remit. The particular scientific questions we seek to answer are driven by our client needs, and before any work is undertaken we confirm that a minimal level of non-animal research has already been conducted to establish that the compounds are safe for use and should, in principal, have the desired effect. Beyond this, our work addresses questions which, by their very nature, cannot be answered using non-animal alternatives. In short, we are providing services which tackle the next stage of the development of medicines, after non-animal research has provided as much information as possible.

Why were they not suitable?

There are no non animal alternatives that can currently replicate the complexity of the mammalian body. Whilst all the non animal experiments provide information that helps understand what a drug might do in an isolated preparation, research in a whole animal is still required. For example, even if a potential treatment for schizophrenia can be shown to activate the correct cell-type grown in culture, the effects in a whole-animal where those cells interact with the rest of the brain can be very different and unpredictable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Because this project is used to provide a service to clients, and the models/data that we are able to provide are extensively used the total numbers are reasonably high. I have estimated the number based of the total usage of animals in the previous project licence.

We have a large number of historical datasets which will guide our decisions about the number of animals required. Where we lack internal data we will refer to external datasets, published data and best practice and following on from initial small scale pilot studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For both rat and mouse studies, historical datasets and power-analysis will be used to ensure all studies maximise the chances of detecting the effects of the treatment, if those effects are present. In this way results, whether positive or negative, should be conclusive, minimising the necessity of repeating studies. We have access to a statistician when required and will continuously monitor group sizes and modify as appropriate.

Where possible, we will use a "Latin-square" crossover study design in which all animals receive all treatments, so that each animal can serve as its own control. This can dramatically reduce the number of animals required for confidence in the statistical analyses, but may not be possible for some protocols. Where possible, animals will be used for multiple studies (such as surgically prepared animals), within the defined limits specified in the licence.

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 when designing new studies to ensure the minimum number are used with the minimal amount of suffering.

Best practice and good experimental design will always be used to ensure quality data and reduce the risk of studies needing to be repeated.

Where possible tissues will be collected from animals at the end of life and used for additional assays.

Animals will be bred for use in the project, to ensure that animals are available at the required age for use, this will potentially reduce the overall number of animals bred.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
The models used in the project have been extensively used to identify candidate drug molecules and are the lowest severity that will allow decision making data to be obtained. These models are also selected based on their translational potential – for example, their face validity for human conditions.

Where pain models are used, the insult is kept to the minimum required to show a pain response, with suffering, distress and lasting harm kept to a minimum. For example, animals receiving nerve injury are placed onto an environmental enrichment protocol, where the enrichment is changed on a regular basis (approximately 2-3 days) with different objects/enrichment to help reduce the incidence of autotomy (toe chewing).

For all pain studies we will continue to encourage the use of spontaneous, non-evoked behaviours and non-invasive (natural) endpoints to reduce the pain and suffering

experienced by the animal such as weight bearing, burrowing and paw volume and any other naturalistic behaviours.

Various models of epilepsy will be used in the project to cover the various types and complex mechanisms in this disease area. Application of electrical current can be used to detect either pro- or anti-convulsant activity, and is therefore useful in detecting an increase in seizure susceptibility, a potential liability associated with several mechanistic approaches of therapeutic interest in this area.

Other models of epilepsy will also be used, such as chemical induced seizures. These models are well validated and doses will be kept to the lowest level required to induce the desired behaviours and to mimic the seizures and biochemical changes seen in human patients. All epilepsy models to be used also have humane endpoints to ensure minimal suffering.

Most models of schizophrenia, Neurodegeneration and cognition used for this project will be mild with minimal pain and suffering to the animal. Some models require administration of chemicals to induce a behavioural deficit similar to that of the condition of the patient such as effects on memory for Alzheimer's and social interaction for schizophrenia, with a non invasive endpoint, assessment of natural behaviours.

Why can’t you use animals that are less sentient?

In drug discovery, in order for an animal model of human disease to be considered valid, it must replicate key aspects of the disease. For neurological conditions this requires a certain degree of genetic and structural similarity. For example it would be pointless to measure hippocampal degeneration - a signpost of Alzheimer's disease - in a species that has no hippocampus. Decades of research have shown that, unlike birds, insects, fish and amphibians, the most ancient cortical structures in rats and mice bear a close anatomical similarity to humans and generate similar patterns of electrical activity. Moreover, almost every gene associated with human disease has an equivalent in rats and mice, making them ideal species for study.

All of our models typically involve a natural response/behaviour such as withdrawal to stimulus or assessment of exploratory behaviours which cannot be detected in an anaesthetised animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All surgical procedures will use aseptic techniques and allow full recovery after surgical procedures. All animals will be provided with sufficient pain relief and post operative care.

Where appropriate, animals will be habituated to environments, equipment and procedures to minimise stress and any lasting harm.

All animals will be provided with environmental enrichment where possible and will be socially housed where possible for the benefit to the animal provided that it does not interfere with the results/data from the studies.

Where possible non evoked/ natural behavioural endpoints will be used, such as burrowing, weight bearing, and behavioural observational scoring systems.

When new models/studies are being developed a small number of pilot studies will be performed to ensure the model delivers the expected results/responses. These will also be used to ensure that the animals experience minimum severity required to give the desired effects needed for the study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to the Home Office and EU guidelines, relevant best practice guidance will be gained from NC3R's (Enrichment and handling techniques, experimental design), LASA (blood sampling volumes, dosing administration volumes, surgical techniques) and GOV.UK (ASRU). We can also use www.bps.org.uk/news-and-policy/new-bps-research-and-ethics-guidance

examples:
https://nc3rs.org.uk/rat-tail-vein-non-surgical, https://www.lasa.co.uk/wp-content/uploads/2018/05/LASA_BAP_BNA_ESSWAP_GP_Behavioural_LAS_Nov13.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to keep abreast of developments in animal welfare by attending scientific meetings, keeping up to date with current literature, attendance to AWERB meetings and maintaining an active member on the AWERB committee.

We will continue to work closely with the NACWO and NVS and seek advice on potential improvements that could be implemented.

We will continue to have an ongoing working relationship with the UK's NC3Rs and will continue to maintain those contacts in an effort to evolve our best practice.

196. Molecular and Cellular Mechanisms of Chronic Pain

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Pain and therapy, Somatosensation, Ion channels and receptors, Signalling, Metabolism

Animal types	Life stages
Mice	adult, neonate, juvenile, aged, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project are to understand how chronic pain is generated, transduced and maintained and reveal the molecules and cells crucial to these 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?

This work will significantly enhance our understanding of the fundamental events and processes underpinning different types of chronic pain. These mechanistic understanding will provide the rationale and foundations for developing more efficacious therapies for transforming the treatment of chronic pain, an unmet clinical need.

What outputs do you think you will see at the end of this project?

We will have a better understanding on how different types of chronic pain are generated and then be maintained for a longer period. We will uncover novel molecules and players

involved in these processes and clarify how they work together to bring about the outcome of chronic pain. This new information and knowledge will be published and made available to the general public, and have great potential to open up new therapeutic opportunities for a better treatment of debilitating chronic pain.

Who or what will benefit from these outputs, and how?

The general public and patients with chronic pain will benefit from the outputs. The research will enhance their understanding of chronic pain and the factors essential to the development of chronic pain. It will impact on pain management of the patients. For example, pain management through diet.

Pharmaceutical companies interested in analgesics development will also benefit from the outputs. We will reveal molecules that could be targeted for developing novel analgesics. We will also test and validate compounds that are effective for chronic pain. These compounds could be further explored for developing efficacious drugs for transforming pain therapy. We will show genetic manipulation of chronic pain which is feasible to be used for gene therapy. If an effective drug and/or a new way of gene therapy were developed based on our outputs, it will benefit millions of patients suffering form chronic pain.

How will you look to maximise the outputs of this work?

We will disseminate our outputs to the general public through science festival, public forum, contributions to magazine, our establishment webpage, press release and other social media. We will directly engage with the patients with chronic pain through one-to-one meeting and online meeting. We will also invite patients to attend our research meeting. Collaboration with our research partners will be another route to publicise our outputs. One of collaborator is a consultant at hospital. His direct link to the patients will facilitate our output dissemination.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult mouse, because: (1) mouse is the lowest organism that models complex diseases in humans and findings from mice are translatable to humans; (2) different types of chronic pain have been successfully reproduced and well-established in mice and pain behaviours in mouse are well characterized; (3) Mice are amenable to genetic manipulations and many gene knocks have been generated in mice and ready for use; (4) Mice are cost-effective and easier to breed and maintain; (4) Pain model and behaviours are more stable in adult mice.

Typically, what will be done to an animal used in your project?

We will generate different types of chronic pain models (e.g. neuropathic pain and inflammatory pain) in normal or genetically altered mice through injection of chemical compounds or surgical operations. A drug or genetic materials will then be delivered to the mice to intervene in the molecules and events crucial to the development of chronic pain. We will monitor pain behaviours of mice and compare pain behaviours before and after interventions. The experiments typically last for 4 weeks, but some will take up to 18 weeks, for example, for diabetic neuropathy.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will mostly experience a moderate level of pain and discomfort typically lasting for 4 weeks and may have reduced mobility and swelling in the paws or joints. Ob/ob mice will likely develop hypercalcaemia and chronic pain. Animals with surgical operations may develop wound infection and bleeding, though these are uncommon. Some animals may also have body weight loss but this should not reach over 20%.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity are mild or moderate. Animals used solely for breeding and control group will be subthreshold or mild severity accounting for about 30%. The remaining animals will be in moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The formation of pain involves collective actions of multiple body systems and numerous cells, many of which even remain unclear. Therefore, neither is it possible to synthesise pain *in vitro* nor possible to directly study pain on humans. Animals are the most practicable platform to achieve our research aims.

Which non-animal alternatives did you consider for use in this project?

We do combine use of immortalised cell lines expressing the molecules of interest to mimic conditions in animals and humans. We use these cultured cells *in vitro* to understand and predict the events and processes that occur in the animals. We will also use tissues from human patients after surgery on condition that ethical consent is approved.

Why were they not suitable?

Cultured cells and human tissues are instrumental in understanding the effects of a molecule or a process or event relevant to pain. However, they do not recapitulate the systemic or global effects of pain that results from pooled actions of millions of cells and numerous molecules and events, most of whom are even unknown, involved in chronic pain. Therefore, cultured cell models are not suitable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate animal numbers based on our current use and the ongoing and future projects that require to maintain 5-7 transgenic mouse lines. These mouse lines need to be crossed to generate novel tissues-specific deletion mutant mice which will then need to be expanded to produce numbers required for both behavioural and tissue research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use power analysis based on published data and previous experience to predict the minimal required number of animals. By using strict statistical prediction in experimental design, we have established optimal numbers of mice needed to reach the 5% significance level, thereby reducing the numbers of subjects used. To reduce experimental variability, littermates of transgenic animals will be used as controls. We will use unbiased experimental design (e.g. randomized block design, blinding) considering animal sex factor difference (we will use either sex of mice). Furthermore, we will use tissues from human patients rather than from animals, serving to further reduce use of animals to a minimal level.

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 heterozygous mice to breed wild-type and gene knockout animals that will be further used for experiments. This breeding scheme makes the most of every genotype of animals, reducing unnecessary waste of animals. We will also carry out pilot experiments to determine the optimal operations, procedures and dug dose schemes before formal experiments. Good practise from the published resources will also be regularly sought to improve the consistence and reliability of our experiments, which should also help to reduce the number of animals to 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use models of different types of chronic pain including inflammatory pain, chronic rheumatoid and osteoarthritis pain, nerve injury-induced neuropathic pain and peripheral neuropathy. These models are well-established in mice and in common use amongst other experimenters, which has the advantage that they have been well validated and are reliable, that pain is well-defined regarding the onset, progression and duration of pain, that they are known to cause minimum discomfort, and that the results we obtain will be readily comparable with the work of other labs. These advantage will maximise our research outputs meanwhile minimising suffering and distress to the animals.

Why can’t you use animals that are less sentient?

Pain is an alerting behaviour warning animals of dangers and threats in the environment. Therefore, pain requires alert actions of well-developed systems and consciousness. During immature life stage, the involved pain systems and the links between systems are not well developed and established. Pain behaviours therefore do not reflect and represent the actual processes in human pain and not useful for our research purpose, though animals may experience less pain at immature life stage. Indeed, there are reports showing that juvenile pain and adult pain utilise different mechanisms. Our research focusses on chronic pain which is more prevalent in adult and older people. Therefore, less sentient animals do not suit our research purpose. For the similar reason, terminally anaesthetised animals are not useful for our research, as pain does not manifest under anaesthetised condition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will closely monitor the general heath conditions of mice including body weight, feeding, drinking, walking and grooming after a procedure such as surgical operation and drug administration at a daily basis for the first 3 days. In the event of an animals displaying abnormal behaviour such as failure to move freely, feed, groom or socialise normally the affected animals will be closely observed and the NVS or NACWO will be consulted. We will further refine and improve the procedures based on our observations and advice of NVS or NACWO.

To recover anaesthetised animals in a procedure, animals will be put in a warm cage and closely monitored during recovery from anaesthesia and daily thereafter. Appropriate analgesics (e.g. NSAID) will be administered to the mice soon after surgery for the first 24 hours of post-surgery. All the pain behaviour tests will be carried out after sufficient habituation of animals aiming to reduce stress to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will observe the guidance on the operation of the Animals (Scientific Procedures) Act 1986 and the ARRIVE guidelines, and follow the LASA guidelines on dosing and surgery. We will also follow the principles of reducing harms and optimising benefit (Harm-Benefit analysis) throughout the conduct of animal research, making sure that our experiments

and procedures are well refined to the best of our knowledge.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To keep abreast of advances in the 3Rs, we will regularly discuss with our animal technicians and manager on potential improvements on our experimental procedures and practice and new development in this area. We will also learn new 3Rs approaches, tools and technologies through attending and watching related webinars and keeping an eye on the NC3Rs website and activities.

197. Immunobiology of leishmaniasis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Leishmaniasis, Immune system, Myeloid cells, Vaccines, Immunotherapy

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?

A key feature of leishmaniasis is the ability of the Leishmania parasite to spread within and between different tissues. This ability to disseminate underpins the pattern of clinical disease, but the mechanisms controlling dissemination are poorly understood. The aims of this project are to understand how Leishmania exploits the immune system to aid its dissemination (including in the presence of co-infections such as malaria), to identify pathways for intervention that limit clinical disease (immuno-therapy) and to further develop vaccine candidates for the treatment and prevention of leishmaniasis (including the conduct of pre-clinical studies supporting clinical trial authorisation).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Leishmaniasis is a globally important parasitic disease with approximately one billion people at risk of infection and over 1 million new cases and ~20,000 deaths reported each year. We currently have no vaccines licenced for use in humans to prevent or treat leishmaniasis. Current drugs have significant side effects and / or are poorly effective against many forms of leishmaniasis. Understanding how Leishmania parasites

disseminate in the body and finding ways to prevent this, using drugs and / or vaccines will lead to dramatic improvements in health and economic development in countries affected by leishmaniasis.

What outputs do you think you will see at the end of this project?

We expect the following outcomes:

publications in scientific journals and in publicly accessible reports that enhance the collective understanding of leishmaniasis by academics, clinicians and the public.

identification of mechanisms of parasite dissemination and interventions (drugs, vaccines) that inhibit this process, leading to reduction in disease prevalence and / or severity of clinical disease.

data supporting the further development of new vaccine candidates, vaccination strategies or immunotherapies into early phase clinical trials.

Who or what will benefit from these outputs, and how?

The benefits and beneficiaries include:

people living in countries with endemic leishmaniasis will have direct health and economic benefits if a preventative or therapeutic vaccine or other immuno-therapy is developed. Our work aims for registration of a new therapeutic vaccine within a 5 year time frame and a prophylactic vaccine within a 5-10 year time frame.

countries with endemic leishmaniasis will have increased prosperity due to improved health of the work force, with benefits seen over the 10-20 year time frame post implementation of a vaccine or new treatment. The scientific community will gain additional understanding of this disease and of immune mechanisms that may also operate in other infectious diseases. The benefits from new knowledge will be both short (12-24 months to first publication) and long (5 years +) term.

How will you look to maximise the outputs of this work?

The project is highly multidisciplinary and international. Animal studies are conducted in parallel with clinical research and clinical trials, allowing an iterative approach. Research collaborations exist in many countries where leishmaniasis is endemic. We have built infrastructure for efficient data sharing. We publish in open access journals and deposit manuscripts prior to publication in pre-print servers to facilitate rapid data exchange. Social media is used to rapidly disseminate key information. Patient and Public Involvement and Engagement is central to our work and will be extended.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents provide the best pre-clinical models to study parasite dissemination and the immune response during leishmaniasis. Mice are the preferred species due to the greater availability of immunological tools. Adult mice of either sex are used, with or without genetic modification or environmental conditioning (e.g. UV exposure; co-infection). Occasionally, young or old mice may be required to address specific questions related to immune system development and / or to assess vaccine or drug efficacy across the life-course.

Our work with mice is complemented by collaborative research on animals conducted outside the UK and with leishmaniasis patients, reflecting a "one health" approach to addressing the problem of leishmaniasis.

Typically, what will be done to an animal used in your project?

Typically animals in this project are used to understand more about the disease process caused by Leishmania parasites and to evaluate new vaccines and therapies. A typical animal (>70% of animals in the Project) might therefore receive five or six injections (of vaccines, drugs or cells) to manipulate its immune system and receive a single injection to establish a Leishmania infection. Alternatively, infection may be established through the bite of Leishmania-infected sand flies. The infection and immune response would be monitored for four – eight weeks by taking a small blood sample every week or by using a special camera to quantify the number of parasites present. No overt signs of disease or changes in behaviour are expected over this time period. After four to eight weeks, the animal would be killed to recover parasites and immune cells for further study. Allowing uninfected sand flies to take a blood meal (one millionth of a litre each) from some animals (<5% of those in the Project) whilst they are anaesthetised is an important way to find out if drugs and vaccines can stop transmission of parasites.

Other procedures or combinations of procedures may be used on some animals. In complex experiments, animals may have a small microchip inserted under the skin to aid identification. <5% of animals in the Project may be co-infected with different parasites or bacteria that also co-infect humans with leishmaniasis and affect immune response. Other animals (<5% of those in the Project) may be exposed to irradiation and have their immune system replaced by a bone marrow transplant. This procedure lasts a few minutes and is usually performed 8-10 weeks prior to infection. Some animals (<10% of those in the Project) will be used to maintain infective stocks of the parasites under study. These experiments last several months, but again without overt clinical signs or impact on the animal behaviour. Some animals (<10% of those in the Project) are used to provide normal blood or tissue (e.g. for in vitro work) and are used without manipulation or within days of an injection.

Experiments are terminated by a humane method of killing. This may be preceded by imaging of tissues exposed by surgery of by using a simple technique called “microdialysis” that allows sampling of body fluids over a few hours.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals used in this project show no outward signs of distress or suffering. Injections produce mild discomfort and some stress associated with handling. Sand fly

bites may cause a minor transient inflammation at the bite site that resolves over a few days. Anaesthesia is used for restraint where needed and recovery from anaesthesia occurs within minutes. Irradiation and bone marrow transplantation produces no overt clinical signs. Infections are always terminated before any severe symptoms occur and in most cases before any clinical symptoms occur. Weight loss can occur over a period of several weeks but will not exceed 20% body weight and in most cases is negligible. Animals show no abnormal behaviour because of the infections, drugs, cells or other substances administered. Cutaneous leishmaniasis causes skin lesions at the site of injection (or sand fly bite) that develop over several weeks, but these do not impede normal behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Infections with Leishmania and other pathogens is "moderate" (40%) All other procedures are “mild” (60%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our research on parasite dissemination requires studies to be performed in the context of complex tissues linked by a circulatory system of lymphatics and blood vessels. This complexity cannot be recapitulated in organ culture or other in vitro systems.

Pre-clinical data supporting immunogenicity and / or efficacy of vaccines and immunotherapies using animal models of disease is required by regulatory authorities prior to clinical trial authorisation. There are no substitutes to conducting vaccination / therapy studies in intact sentient animals due to the complexity of the host pathogen interaction and the dynamic nature of the host immune response. In vitro culture models and mathematical models are not sufficiently advanced to provide data sufficient to support regulatory submissions.

Which non-animal alternatives did you consider for use in this project?

We have an active research program exploiting using mathematical and computational models and these inform some aspects of our work.

We continue to explore new types of organ and in vitro models as they are developed.

We use existing high dimensional data sets in the public domain to supplement, compare with and add value to our data, minimising repetition of experiments conducted by others.

Why were they not suitable?

These approaches fail to provide the appropriate context for understanding parasite dissemination and / or for supporting requests for clinical trial authorisation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimates of animal usage are based on consideration of the scope and duration of funded work, staff workload, balance of experimental and analysis time and the need to ensure adequate training and research planning.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In vaccine studies, we normally only study candidate antigens or delivery systems well advanced in pre-clinical development, using antigen delivery platforms with established safety data in man and applicability to developing country use. Most drugs we study are clinically approved and available for re-purposing.

We use the NC3Rs EDA, coupled with statistical analysis using InVivoStat software and /or additional packages in R. to ensure appropriate and minimal numbers of mice are used to generate statistically meaningful results. Local statistical advice is also available.

Tissues collected from animals used in in past experiments may be used in new experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We reduce breeding of genetically-altered and / or mutant strains by careful planning of experiments, tissue / cell banking (creating a curated tissue bank for cross reference and future analysis) and through high density data collection (e.g. through imaging and omics approaches). Material from our tissue banks may be made available to other investigators for independent studies on inflammation and immunity.
Whole body non-invasive imaging is used to generate longitudinal data from small cohorts of mice and other imaging techniques allow cells rather than mice to become the experimental unit (reducing animal numbers required for statistical analysis).

Data are used to develop mathematical models which can be used to inform on experimental design.

Experiments are often conducted in parallel to allow re-use of tissues / cells between experiments, reducing the number of mice needed only to supply tissues and cells.

Our active research into human disease allows us to restrict animal studies to those questions of direct translational relevance

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are most-commonly used as the lowest mammalian species in which the various infection models used in this Project can be studied. Mice also provide the opportunity to learn the mechanistic basis of host protection and pathology in a way unrivalled by other models.

Most studies will use standard strains of mice to understand parasite dissemination and the host immune response to infection. Leishmaniasis is not usually lethal in mice, reducing the possibility of severe outcomes compared to other rodent models.

A rigidly enforced health policy exists in the animal facility, including screening for additional bacterial pathogens beyond standard guidelines. Any changes in breeding success, animal behaviour or any unexpected deaths are rigorously investigated.

We minimise pain suffering and distress by early termination of most infection experiments, for example before significant hepatosplenomegaly or cutaneous lesions develop. Strict adherence to the use of appropriate humane endpoints and adverse event scoring will be observed. We restrict the total number of injections and anaesthesia sessions per animal as advised by the NVS.

Any animal showing behaviour that is unexpected will be killed by a schedule 1 method and examined post-mortem and the NACWO and NVS are informed.

Fluorochrome-expressing genetically-altered mice are used to minimise the need for additional animals to provide populations of cells for studying cell trafficking.

Any surgical techniques are practiced on dead animals until competency is achieved and then pilot studies are conducted under NVS supervision.

Based on previous experience we have developed new protocols to minimise animal use in maintain sand flies and conducting infectious challenge experiments

To ensure minimal UV damage to skin, we use advanced instruments to monitor redness and changes to skin structure.

Why can’t you use animals that are less sentient?

The mouse is the lowest sentient mammalian species for the study of Leishmania parasites. Leishmania can infected macrophages from zebrafish but this is not a realistic model of infection in vivo given differences in the circulatory systems, tissue organisation and immune response. Lower vertebrate species are not infected with species of Leishmania that infect humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We adhere to the rolling program of advances in husbandry introduced by the animal facility e.g. implementation of environmental enrichment, changes in handling methods and changes in the policy regarding re-use of needles.

Increased monitoring of animals under procedure is being introduced to minimise any adverse events e.g. during long term parasite passage in immunodeficient mice.
Body score conditioning sheets and grimace scoring will be used to monitor the condition of animals, where appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow ARRIVE2 guidelines for reporting of experimental results. The NC3Rs EDA and associated webinars and publications are used to ensure robust experimental design (in conjunction with "The Design and Statistical Analysis of Animal Experiments; Bate and Clark (2014)"

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly consider and follow new approaches in experimental design, new technical innovations that may reduce animal usage or increase data acquisition / robustness and ethical issues related to animal research. Where appropriate, amendments will be sought to introduce or pilot new techniques scoped from the NC3Rs website or elsewhere. Regular lab meetings and investigator meetings (e.g. Animal Facility Users' meetings) provide a forum for discussion and implementation.

198. Cell-cell adhesion signalling 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

retina, vasculature, aging, inflammation, diabetes

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Cells in endothelia and epithelia are joined by adhesion complexes that regulate how these tissues function and form barriers that separate different compartments such as the blood from the surrounding tissue. Our aims are to elucidate how adhesion complexes are deregulated in disease and to establish new therapeutic methods exploiting such knowledge.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Cells in epithelial and endothelial tissues are joined to each other by intercellular adhesion complexes. One of these complexes is called tight junction (TJ). TJ are essential for

epithelial and endothelial barrier formation. They also signal to the cells to guide cell behaviour and function.
Pathological processes affecting endothelial and epithelial cells in the retina are the major causes of blindness in the developed world. Visual impairment has major consequences for the quality of life of the affected individuals and represents a significant burden to society. Our research on TJ functions in ocular endothelial and epithelial cells in mouse models thus addresses a major clinical need.

Controlling inflammation and aging are a major emphasis of our research. Inflammatory and aging diseases affecting the eye are very common and mechanistically related inflammatory diseases affect other organs such as lung, liver and intestine. The socioeconomic costs of such diseases are considerable and there remains a real need for developing new therapies. Our programme to understand the role of TJ proteins, to identify specific ways to regulate TJ functions, and to test specific inhibitors for TJ associated proteins such GEF-H1 will directly address this need.

Therefore, the work to be undertaken is important as it will enable us to understand the functions of TJ components in health and disease, and it will support the development of new therapeutic approaches to treat wide-spread and debilitating diseases and, thereby, addressing a clinical need.

What outputs do you think you will see at the end of this project?

Outputs will include information about how cell-cell adhesion and tissue barrier formation is coordinated by TJ proteins during development, adult aging and pathological stress conditions. This is needed to understand the basic mechanisms of how aging and diseases such as diabetes impact on the retina. Such new information will be published in research papers. We will also test new therapeutic approaches. This will lead to outputs in the form of information on how to apply such new therapies and on the potential of particular therapeutics. Such information will also lead to publications but will also provide the basis of clinical trials to test such therapies and associated therapeutics in humans. Hence, the information expected will contribute to the development of new therapies and new products to be used in such therapies.

Who or what will benefit from these outputs, and how?

The generation of new knowledge and the development of new therapeutic approaches will benefit scientists and clinicians in academia and industry who work on the development of new therapies, as well as patients who need improved and more effective therapies.

The benefit on other scientists and clinicians will be more immediate as the expected output will directly impact on their work. The expected information will help them to design new therapeutic strategies to be tested first in the laboratory. Information about new therapeutics and the compounds themselves will help clinicians to design clinical trials to test them in humans.

In the longer term, the outcomes are expected to benefit patients and society as a whole. The new therapeutics by leading to better treatments will impact on patients health, which, in turn, will reduce socioeconomic costs.

How will you look to maximise the outputs of this work?

This project will investigate the physiological and pathological relevance of molecular mechanisms by which TJs regulate epithelial and endothelial tissue functions as well as their role in disease initiation and progression during inflammatory, aging and degenerative conditions. We will also develop new therapeutics.

To maximise the outputs we will

disseminate our results in publications, meetings and workshops to reach other scientists and clinicians;

build on and further extend collaborations with clinical colleagues to accelerate the development of clinical applications based on our research and expected products;

seek advice and support for Intellectual Property (IP) and commercial development, which will support funding of follow-up research;

and engage with the general public and patients to inform them about new research developments pointing to new therapies and for a consultation to support the design of new clinical approaches.

Our approach is built on extensive (in a test tube or elsewhere outside a living organism) in vitro work that we have performed during the last 20 years using different types of cultured cell models that have allowed us to identify the most promising molecular targets. However, it is possible that mechanisms identified in vitro are of lesser importance in vivo (in a living organism). It will thus be important to disseminate positive as well as negative results.

Species and numbers of animals expected to be used

- Mice: 6000
- Rats: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project is built on extensive in vitro work on the structure and function of TJ in epithelial and endothelial cells that has been performed in the past and that will be continued to complement and support the in vivo experiments. The data and insights gained from those in vitro studies are providing the necessary knowledge base to design meaningful in vivo studies in order to establish the functional relevance of TJ-associated signalling mechanisms in normal development and clinically relevant animal disease models, as well as to determine the potential of therapeutics targeting such signalling protein.

We will make use of well-established and validated mouse and rat disease models for which we have experienced collaborators within UCL and elsewhere who can advise on experimental design and animal manipulations. This will allow us to base our studies on

years of experience that have led to refined protocols that enable the collection of meaningful results with as few animals as possible.
Moreover, we will be able to compare our results with the existing literature to support conclusions about the importance of our findings.

The mouse and rat disease models (neonatal, juvenile, adult) have been refined to understand human diseases. These will be used to determine the effectiveness of therapeutics that we have developed in in vitro experiments to attenuate tissue inflammation and degeneration due to conditions such as diabetes, or age-related and inherited forms of retinal degeneration. We will further employ genetic mouse models (embryonic, neonatal, juvenile, adult) deficient in specific junctional proteins to determine how far their associated signalling mechanisms are important for development and tissue maintenance, and how they interact with disease pathways. The use of animals at different life stages is important as analysis of developmental defects will require adult and neonatal animals, and the disease models are based on juvenile and adult animals.

Typically, what will be done to an animal used in your project?

Wild type and genetically altered animals that are deficient or conditionally deficient in specific genes encoding TJ components to be studied will be bred using protocol 1 and 8. These mice will then be studied using protocols 2 to 9. We will also purchase and maintain wild type rats and strains with naturally occurring mutations that lead to spontaneous retinal degeneration.

Protocol 1 is to maintain wild type, genetically modified and naturally occurring variants of mouse and rat strains and to cross-breed strains to generate novel combinations of genetic alterations. Offspring carrying desired alleles and their wild type littermates will be used for analysis at various developmental stages and at different ages after birth to study the role of TJ proteins in healthy and disease animal models.

Protocol 2 is to characterise and validate therapeutic approaches to rescue pathological responses during ocular surface inflammation; e.g., allergic conjunctivitis or dry eye. To induce ocular surface inflammation, we will use only one of the optional protocols. If we use an allergen (either ovalbumin (OVA) or short ragweed (SRW) pollen), mice will be sensitised by intraperitoneal injections. Disease induction, assessments and treatments will last up to 3 months.

Protocol 3 involves refined models for experimental-induced uveitis (infectious and autoimmune). To induce uveitis, we will use only one of the optional protocols, by intraperitoneal injections of retinal antigen or intraocular injection of LPS (Lipopolysaccharides are large molecules found in the outer membrane of bacteria). Only one of the methods to induce uveitis will be used for a specific animal. Depending on the method used, disease induction, assessments and treatments will last up to 4 months.

Protocol 4 is a model of neovascularisation during development and pathological conditions to characterise junctional signalling molecules and test therapeutics. Offspring mice carrying desired alleles will be used at various developmental stages and at different ages after birth. Neovascularization will be induced by exposure of animals to high oxygen levels prior to incubation under normal oxygen, or exposure to strong light sources. Disease induction, assessments and treatments will last up to 9 weeks, depending on the method used. Only one method will be used for a specific animal.
Protocol 5 is to model diseases affecting the retinal vasculature. Ischemia and increased permeability of the retina, as well as corneal or retinal wounding will be induced by laser

ablation. Diabetes will be induced in mice or rats by intraperitoneal injections of Streptozotocin (STZ is an antineoplastic agent that is toxic to the pancreas in mammals) or high fat diet. Diabetes development will be followed by taking blood samples to analyse glucose levels and eye assessments to determine the role of a given TJ protein or potency of therapeutics for up to 15 months.

Protocol 6 is the generation of specific monoclonal antibodies for junctional proteins. We will do intraperitoneal injections of an antigen every 30-45 days, 3-4 times, and collect blood samples until we detect the production of specific antibodies.

Protocol 7 is to assess whether novel therapeutics rescue permeability triggered by common disease mediators (e.g., LPS, VEGF, TNFalpha, etc) in an assay called Miles assays.

Protocol 8 is a breeding protocol to allow to assess those GA strains that show a moderate severity. The protocol will allow us to assess eye functions in these GA (genetically altered) strains to decide in which protocol a new GA strain will be analysed.

Protocol 9 is to test experimental gene therapy approaches to rescue retinal function. We will manipulate expression of TJ or associated signalling proteins by ocular injection of viral particles into wild type and GA mice, or rats and evaluate if and how retinal function is rescued by different types of eye assessments, e.g. acquiring electroretinograms (ERGs) and by ophthalmic examination through non-invasive fundoscopy or laser scanning ophthalmoscopy for up to 15 months.

What are the expected impacts and/or adverse effects for the animals during your project?

In Protocol 1, mild, we do not expect pain, weight loss, tumours, or abnormal behaviour. In the majority of animals covered by this protocol activation of conditional mutations will be harmless. However, occasionally it will not be known in advance whether the activated transgene will cause any harm to the animal.

In Protocol 2, moderate, intraperitoneal sensitisation is expected to induce low adverse effects. Ocular surface allergic conjunctivitis leads to mild to moderate adverse effects leading to eye swelling, tearing and mucus production.

In Protocol 3, moderate, uveitis is usually well tolerated (>99%) by the animals. Animals with uveitis exhibit transient mild inflammation of the eye with onset about 9 days after disease induction. Eyes will appear cloudy at this point, due to white blood cell infiltration into the eye. This usually completely resolves by 3-4 weeks after disease inoculation. This may result in sight loss due to photoreceptor damage. Vision is not the major sense by which small rodents perceive their environment and most animals will be killed after 14 days. The loss of sight does not affect their normal behaviour and feeding but animals with uveitis will have reachable food at all times on the cage base as well as in the hopper.

In Protocol 4 and 5, moderate, disturbed retinal development is anticipated to be pain free because the retina (like the brain) lacks pain receptors. Blindness may be a potential outcome, but mice kept in captivity make very limited use of their visual senses, which is illustrated by the fact that genetically blind mice thrive and breed as well as normal mice in captivity. Hyperoxia exposure can have toxic effects on adult mice and, therefore, mothers are only exposed for a maximum of 5 days to hyperoxia. In Protocol 5: mice do not become ill as quickly as diabetic humans. Adverse effects can be observed at sites of

administration of Streptozotocin; thus, a particular focus will be the monitoring for infections and wounds.

In Protocol 6, moderate, most antigens can be injected without causing adverse effects on the animals' wellbeing. However, immunizations may cause mild responses. As a consequence of Freund's complete adjuvant injection granulomas may be formed at the site of adjuvant injection. Injection in the flank reduces these adverse reactions and the animals show no untoward side effects as a result of this protocol.

In Protocol 7, moderate, the effects of hyperpermeability-inducing agents in the skin induce mild to moderate adverse effects leading to swelling and itching.

In Protocol 8, moderate, the adverse effects expected to result from the genetic alterations are unknown. Animals bred on moderate severity limit protocols are expected to develop a phenotype that is sufficient to cause a disease comparable with the moderate severity description (as Home Office document Advisory notes on recording and reporting the actual severity of regulated procedures). The disease is not expected to be fatal, and animals will be killed before any disease becomes life- threatening.

In Protocol 9, moderate, sequelae from intraocular injections are generally temporary and cause little discomfort. Local antibiotic/steroid ointment and aseptic injection techniques will prevent infections potentially associated with intraocular injections. Placement of ERG electrodes may occasionally result in infections which will be prevented through local antibiotic/steroid ointment and good aseptic technique.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 1 is Mild severity (60%).

Protocol 2 to 9 are of Moderate severity (90%).

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 have studied cellular functions of TJ proteins in cultured cells in great detail and will continue to do so in the future. Our strategy is to elucidate the molecular mechanisms and structure/function properties of TJ proteins in vitro so that in vivo experiments can be designed carefully and with sufficient insight to keep animal numbers to a minimum.

The mechanisms to be analysed are processes for which we have generated inhibitors as potential therapeutic tools. These inhibitors have been characterised in vitro extensively using cell lines as well as disease models based on primary cultures of endothelial and retinal pigment epithelium (RPE) cells, as well as patient-derived cells; however, before they can be tested in clinical trials, animal experiments are essential to establish that such therapeutics are as effective in vivo as they are in vitro.

We will also analyse the role of junctional signalling mechanisms during development and organ function as there are no alternative to animal models to model complex tissues and organs such as the eye, or to determine effects of retinal dysfunction on visual function.

We will focus on TJ components that we have analysed in great detail in vitro and have established the signalling mechanisms they control in cultured cells. However, an in vivo analysis is now needed to understand more complex in vivo roles such as how such junctional signalling mechanisms regulate complex morphodynamic processes of developing tissues, how different cell types cooperate in organ development and function, how junctional barrier defects lead to tissue and organ malfunction and disease, and how TJ-associated signalling mechanisms regulate neovascularization. Such processes cannot be analysed in vitro only.

Mice and rats are the species best suited for the planned studies because their cells and tissues are similar to the human ones, powerful genetic tools have been developed, and appropriate disease models have been established and well characterised.

Which non-animal alternatives did you consider for use in this project?

All our in vivo studies are based on extensive in vitro data and we will continue to refine our understanding of the junctional proteins using different cell lines and primary culture models. We are also employing models based on induced pluripotent stem cells derived from patients carrying retinal disease genes to model human ocular diseases. Additionally, we will include the analysis of primary cultures of cells from genetically modified mice to reduce pain and discomfort on live animals.

Why were they not suitable?

The in vitro models are limited to 1-2 cell types and cannot recapitulate complex eye diseases and effects of cellular defects in the retina on visual function. Animals are therefore necessary to determine the role of new mechanisms identified in cells in culture and validated in in vitro ocular disease models of eye diseases such as uveitis and diabetes. Unfortunately, whole eyes and retinas cannot yet be modelled in the laboratory without animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

All estimations of animal numbers are based on results from similar studies in the past as well as pilots experiments that allow us to identify differences between conditions using appropriate statistics. The minimal magnitude of such differences (i.e., effect size) have been predefined so that the number of animals calculated is neither too small to detect such differences nor too large to avoid unnecessary use of animals. The total number of animals for specific protocols was then estimated by multiplying these numbers with the number of expected studies to be performed.

For protocols that include different assessments, the main readouts have first been determined to calculate required animal numbers. For example, in the analysis of vision in live animals, the ERG measurements are the most important assay as they allow conclusions about retinal function; hence, we calculated how many animals are required to detect expected effects in ERG measurements. We also considered the number of animals required to perform meaningful post-mortem tests to ensure that conclusive results will be obtained.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We based our calculation on previous experiments, pilot studies and published results that allow estimation of all analysis methods to be used. Additionally, we use the Experimental Design Assistant (EDA) online tool from the NC3Rs, designed to guide researchers through the design of their experiments, to ensure that we use the minimum number of animals consistent with our scientific objectives, methods to reduce subjective bias, and appropriate statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Prior to analysing newly developed therapeutics and the function of junctional proteins, we have and will continue to perform extensive in vitro experiments with cell lines and primary cultures originating from different cell types and tissues.

When we use animals, we will first carry out pilot experiments to test the best experimental settings. Depending on the type of question to be addressed, the pilot experiments will include control and experimental groups with up to 2 animals per group. These will then be translated into full scale studies if satisfactory results are gained and repeated in sufficient numbers, assessing both qualitative and quantitative outcomes to ensure reproducibility and statistical significance. A variety of approaches (in situ hybridization, antibody staining and genetic labelling) will be used to assay the effects on the specific cells. Experimental repeats are primarily aimed at ruling out random events, artefacts and errors. It is therefore generally possible to establish the effect of a given manipulation by comparing a small number of experimental animals (typically around three to five) with non-treated or wild-type controls, thus avoiding experiments with large cohorts of mice.

The serial retinal imaging of animals (e.g., by angiography) and measuring retinal function by ERG reduces the need of killing animals at every experimental time point and facilitates the selection of optimal timepoints to terminate the animals. The mouse numbers used at each age and/or experimental condition will thus be kept at a minimum by assaying different parameters in the same mice and by keeping the numbers to the minimum required for statistical significance,

For the maintenance and generation of genetically modified strains, the colonies will be kept at the minimum size required to generate sufficient animals of appropriate genotypes for statistically sound experimentation and to keep the colonies stable. The breeding scheme will minimise the generation of harmful mutants and involves breeding heterozygous animals that have no or only mild symptoms (e.g., p114RhoGEF deficient homozygotes are not viable). It is therefore unavoidable that mice are generated that do not carry the wanted alleles. They will be culled without having undergone any experimental procedures or used as controls in experiments to reduce the breeding of control animals. In any case, we will be conducting our experiments so that we will be able to publish according to the ARRIVE guidelines [https://www.nc3rs.org.uk/arrive-guidelines] and will use randomisation and blinding where appropriate to avoid bias.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 design breeding strategies that reduce animals with deleterious mutations. Postnatal animals will be health monitored on a daily basis or, if an unknown strain is analysed, more frequently. If animals show signs of suffering due to the genetic mutation or to an unexpected strong response in a disease model, they will be immediately humanely culled using a schedule one method.

A mammalian experimental model is important to bridge the gap between in vitro studies and humans. Considering the existing knowledge and available strains and experimental tools, mice provide the most refined model and allow us to make use of strains and experimental tools that have been generated as part of international efforts to generate genetic models of the entire mammalian genome (e.g., p114RhoGEF knockouts). Moreover, we can use conditional transgene technology that reduces the number of genetically altered mice actually suffering from their mutations as both the effector and the responder strain are symptom-free. When possible, inducible systems will be used as the offspring from responder-effector crosses from pairs that are symptom-free. Only administration of the activating substance (tamoxifen) will activate the mutation in such offspring from inducible cre recombinase mice. This means that the time window of transgene activation is much shorter and can be followed along a defined time window to identify the type and level of the phenotype, which reduces the impact on the mouse with regards to welfare. As specific cell types can be targeted at specific times, more precise questions can be asked and convolution of the results by the presence of different affected cell types can be reduced.

The junctional proteins and therapeutics to be analysed have all been extensively characterised in vitro (such analyses will be further extended); hence, experiments can be planned with disease models that involve these signalling pathways. As the disease models are validated and used widely, we will be able to use well established quantitative assays and can easily compare our results with the literature.

Refined Protocols are:

Protocols 1 and 8 are for breeding and maintenance (https://www.gov.uk/government/publications/animal-testing-and-research-improve-your-project- licence-application). The use of tamoxifen has been well-established (Feil S, et al Methods Mol Biol. 2009;530:343).

Protocol 2, eye allergy

We will use models to induce allergic eye disease that have been established by different laboratories over many years. Hence, refined protocols have been established that keep the number of animals required at a minimum.

Protocol 3, Models of experimental retinal inflammation

We will induce different approaches to induce retinal inflammation by injecting animals. These methods have been refined over many years. The retina lacks pain receptors and mice to do not require vision to live in captivity. Hence, uveitis is an ideal model to study inflammatory diseases as it keeps discomfort low.

Protocol 4, Characterization of vascular development and degeneration

Animals will be kept at different oxygen levels to manipulate blood vessel growth. This model used for many years; hence, precise times are known and will be adhered to induce the desired effects without causing harm to animals. The model is well accepted for the type of studies to be performed and closely reflects a human disease caused by the same conditions.

Protocol 5, Models of retinal vascular diseases

Protocols to induce diabetes and acute inflammation will be used that have been refined over many years by different laboratories. Hence, they are well accepted and sufficient knowledge is available to keep animal number as low as possible. Animals will be injected with reagents to induce diabetes or inflammation. Disease progression is well understood and measures will be in place to reduce suffering.

Protocol 6, Production of hybridoma cell lines

High-quality antibodies can reduce the number of animals required for experiments as they enable more specific and reproducible detection of relevant antigens. Mouse hybridoma generated from immunised animals is still the best way of generating such antibodies. Once such cell lines have been generated, unlimited amounts of antibodies can be produced without requiring further animals.

Protocol 7, Vascular leakage or permeability Miles assays are excellent to provide quantitative readouts for vessel permeability.

Protocol 9, Vector/stem cells/pharmacological agent eye administration
The protocol to be used to test therapeutics and the importance of mechanisms identified in vitro closely follows the methods used for human therapy. Hence, results obtained can be easily translated to clinical applications. The procedure has been pioneered at our Institute and, hence, we can build our studies on many years of experience and know-how.

Animals will be anaesthetized for retinal injections. Surgery will be done aseptically (following HO Minimum Standards for Aseptic Surgery and LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery http://www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf). Animals will then be followed up with live imaging methods to determine effects on the retina and visual functions. All these methods are used throughout the project and refined methods have been established to reduce the suffering of animals.

Why can't you use animals that are less sentient?

Unfortunately, eye diseases like diabetes and uveitis have to be studied in animals that have retinas that closely resemble those of humans to be able to make conclusions relevant for humans. Consequently, novel therapeutics have to be tested in mammalian models like mice and rats to ensure their relevance for human disease and to fulfil regulatory requirements for clinical trials.

Many of the experiments require disease models that require weeks or even months to develop. Such experiments cannot be performed in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Newly generated genetically modified strains are routinely monitored more frequently to identify early whether mutations lead to unexpected suffering and, if yes, appropriate measures are taken. Similarly, we carefully monitor animals after procedures with the aim to reduce pain. For example, in procedures that require full anaesthesia, animals are monitored until they completely recovered and are then provided with soft wet food. Animals are also monitored the subsequent morning. If they do show the lasting impact of the anaesthetic, they are terminated using a Schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow:

RSPCA and LASA, 2015, Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies. A report by the RSPCA Research Animals Department and LASA Education, Training and Ethics Section. (M. Jennings ed.)

PDF document available for download from www.rspca.org.uk/ethicalreview and www.lasa.co.uk/publications.html

The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines are intended to improve the reporting of research using animals – maximising information published and minimising unnecessary studies (https://www.nc3rs.org.uk/arrive-animal-research-reporting-vivo-experiments).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly visit the relevant websites of such as https://nc3rs.org.uk/the-3rs#replacement to implement advances during the project that can be more effective to

obtain useful results and reduce animal suffering. There are also regular e-mails and meetings to disseminate the principles of the 3Rs that inform us about new developments.

199. Imaging and radiation treatment of cancer

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
  - 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

Radiotherapy, Cancer, Radionuclide, hypoxia, Antibody

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 overarching aim of this project is to improve the outcome of cancer patients by enhancing the effectiveness of radiotherapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Radiotherapy is a highly effective therapy used in the treatment of 50% of patients with cancer. There are several different types of radiotherapy. External beam radiotherapy is applied in a radiotherapy department using an external radiation source which is rotated around the patient producing a series of radiation beams that intersect in the cancer. This means that the cancer receives a large dose but the surrounding normal tissue - organs

etc - receive a smaller dose. The greater the dose that the cancer receives, the greater the chances that it will be destroyed. However the dose of radiation that can be given is limited by damage to normal tissue.
Targeted radiotherapy (TR) which is used to treat lymphoma and prostate cancer and is being rolled out to other cancer types due to its proven effectiveness. Cancer cells have on their surface, markers that are not found or are found in far less amounts in normal cells. These markers can be targeted by highly specific drugs. These drugs only bind to their selected marker. TR involves drugs attached to radioactive atoms (radionuclides) that specifically target these markers and irradiate them at close range. This type of radiotherapy is very specific and will also destroy the secondary cancers (where the main cancer releases cells that set up many new cancers throughout the patient). However cancers contain many cells which vary in the number of markers that they have on their surface - this is called heterogeneity and diminishes the effectiveness of TR as not all parts of a cancer will receive the radiation from a TR radioactive drug.

The project will address tumour heterogeneity by altering the drug so it targets several markers and by using several different radioactive atoms that irradiate cells nearby and at a distance.

As cancers grow their blood supply does not grow with them in a well co-ordinated way so they have regions within them that do not receive much oxygen - these areas are called hypoxic. Radiotherapy is much less effective at killing cancers that have large hypoxic regions. By identifying markers on hypoxic cells we can target these with TR to boost the radiation dose to these cells.

The effectiveness of targeted radiotherapy in a patient can be measured by giving the patient a version of the TR agent with an imaging beacon (another type of radioactive atom that is far less damaging to tissues) that distributes in the patient before a therapy version. The patient is imaged and the amount of RT agent measured. This project will also design and test imaging and therapy versions of targeted radiotherapy agents.

What outputs do you think you will see at the end of this project?

The work from these projects will be published in high quality cancer and radiobiology journals.

Optimisation of radioactive cancer marker-targeted drugs for cancer patients receiving targeted radiotherapy (TR).

To demonstrate that imaging can be used to predict if a TR compound will localise in cancer tissue and be effective in a patient before a therapeutic version of the compound is administered.

Who or what will benefit from these outputs, and how?

Short term - increased knowledge in developing radiotherapy-based drugs which will be published Long term potential benefits:
Improved response of cancer patients treated with targeted radiotherapy by optimising the radioactive drugs administered to patients that seek out and destroy their cancers: Current TR compounds have one type of radioactive atom attached and targets one type of cancer marker. Cancers are heterogenous in the expression of cancer markers between different areas in a single cancer. There are also blood flow issues affecting where the TR molecules go to within the cancer. To overcome this we are developing TR compounds

that have two types of radioactive atom that will emit lethal particles both nearby and at a distance from where the TR molecules localises within the cancer. We are also targeting two cancer cell markers. Our hypothesis is that this will enable a better distribution of lethal radiation within the cancer so that all the cancer receives lethal radiation and no part of the cancer is left to regrow.

Reducing morbidity from radiation damage to normal tissues: TR molecules are damaging to normal tissue if they do not preferentially go to the cancers in the patient. However it is possible to determine where the TR compounds go by labelling them with an imaging radioactive atom (which is far less damaging) and imaging the patient and calculating how much goes to each tissue. From the image the radiation dose delivered to each organ and the tumour can be determined (this is known as dosimetry). If the dosimetry indicates that a patient the benefit/harm ratio is high the patient can be given the TR compound this time with the lethal radioactive atom attached. It is also possible to tailor the amount of TR molecule that is given to the patient to reduce normal tissue damage.

How will you look to maximise the outputs of this work?

Targeted radiotherapy (TR) uses a range of radioactive atoms which produce different forms of radiation. One of the challenges with researching and clinical delivery of TR is obtaining the radioactive atoms. This work will involve collaboration with the a nuclear institute which will optimise the delivery of several clinically useful radionuclides. Collaboration with a Government nuclear laboratory will ensure a source of 212Pb which is a very effective cancer killing radioactive atom. Ensuring a broad stakeholder audience for the developments from this project will be assured through interaction with the CRUK steering group on targeted radiotherapy.

It is envisaged that the project work described here will provide useful methodology for advancing radiotherapy. However it is possible that not all approaches will prove to be effective. These less positive outcomes will still be published.

Species and numbers of animals expected to be used

- Mice: 800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The cancer models that are required for this work are cancers growing in nude adult mice or rats. These animals are the least sentient organism in which cancers can be grown that can receive radioactive drugs and be monitorable with medical imaging modalities such as PET. The cancers (xenografts) need to be of a large enough size to contain emissions of radioactive atom which can be 1cm in distance so a 1.2cm3 (1.0cm diameter) is required.
Typically, what will be done to an animal used in your project?

Cancers will be generated in nude mice by injected cancer cells or pieces of cancer into one of the flanks of anaesthetised animals. Animals are anaesthetised which is carried out by placing them in a tank filled with an anaesthetic gas. The animals are kept

anaesthetised via delivery of anaesthetic gas through a face mask. Insertion of cancer cells via a needle by insertion of a piece of tumour into a flank via a tube with an outer diameter of about 3mm. In both cases the needle is inserted just under the skin (sub-cutaneous) and only in one flank. The animal will not be aware of the procedure until regaining consciousness - generally mice do not demonstrate any awareness that the procedure has taken place - rapidly returning to feeding.

Cancer growth will be monitored by regular inspection of the cancer site. The cancer will not be allowed to get any larger than 1.2 cm^3 volume (about the size of a standard marrowfat pea). This size will not interfere with the animals movement or cause it distress. This volume is to accommodate radiation from high-energy radiation-emitting radioactive atoms which travel up to 1.0 cm distant from the radioactive atoms. It is envisaged that for most of the studies using lower energy emitting radioactive atoms (where the radiation range is low) outlined in this PPL the maximum volume will be <1cm^3 volume (about the size of a garden pea). Mice do not demonstrate any awareness of the growing cancer. During cancer growth dimension measurements are taken using calipers. When handled during these measurements the mice are curious and do not demonstrate any signs of stress. The animal is also weighed by placing on a top-tray balance. When the cancer is a suitable size for experiments the animal might be injected with a radioactive drug that seeks out and binds to the cancer. It is unlikely that the mice will be aware that they have been injected as these drugs do not irritate and are suspended in a a non-irritating medium that has a neutral pH. Apart from decreasing the growth rate of the cancer the substances are unlikely to have any effect on the mice. The animal may then be killed by schedule 1 under anaesthesia between 5 minutes and several days after injection. Alternatively the effect of the radioactive drug on the growth of the cancer may be monitored over a period of several weeks.

For some experiments the animals may be imaged after injection of a radioactive substance. Imaging involves anaesthetising the animal as described above. The mouse in then placed in an animal PET scanner. During imaging under anaesthesia the animal will be placed on a heating pad to ensure the animals is kept warm. It is likely that mouse core temperature will be monitored by a rectal thermometer. At the end of the imaging session the animal is either euthanised by a schedule 1 method or allowed to recover. Some animals are scanned on two occasions with at least a 4h gap between during which time the animal will have fully recovered and be exhibiting normal behaviour.

If animals do not recover consciousness within 1h they are euthanised by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice develop tumours which are unlikely to spread beyond the site of injection. Mice will be closely monitored and scoring system developed and humane end points identified and updated as required.

For some of the studies we will require a large blood sample which can most humanely be acquired by cardiac puncture under terminal anaesthesia.

If any of the procedures result in pain as evidenced by apparent facial expression (Facial expressions of pain) analgesia may be given as a first line but if this is insufficient to alleviate pain the animal would be euthanised by a schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The level of severity is expected to be moderate or more commonly mild for any of the procedures detailed in this project.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Targeted radiotherapy uses radioactive atoms which emit radiation of different energies. Radioactive atoms that produce low energy radiation kill nearby cells. Radioactive atoms producing high energy radiation travels further and kills cells at a distance. One part of this project is to overcome the problem of heterogeneity in the distribution of cell kill within the tumour by combining different energy atoms to kill cells close by and at a distance. The distance that we need to observe cell binding and cell kill is several mm. We can only demonstrate the benefit of these systems in 3D using cancers growing in living organisms.

This project will produce optimised targeted radiotherapy drugs which will be aimed at clinical translation. Before any radiolabelled drug is administered to a patient it must be tested in animals and organ dosimetry determined - (where the TR compounds go and how much radiation they carry into organs) by labelling them with an imaging radioactive atom (which is far less damaging) and imaging the patient and calculating how much goes to each tissue.

Which non-animal alternatives did you consider for use in this project?

The only alternatives are tumour spheroids which are 3D models of cancer grown in vitro.

Why were they not suitable?

Tumour spheroids lack a vascular system so only grow to a few hundred micro-metres in size which are far too small for cancer heterogeneity studies.

Exploring imaging methods to characterise tumours requires an in vivo tumour 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?

Radiation-sensitive cancer cells will be used to derive the xenografts to achieve the greatest effect of radiation on tumour growth. Inbred animals of similar age will be used for each study to reduce variation. The basis of most of the work in this PPL is developing radiopharmaceuticals that can be used in man. To achieve clinical translation these compounds must be preclinically testing in animal tumour models and bio-distribution determined. The uptake and tumour distribution of radiopharmaceuticals is also dependent on a blood supply.

Similar work in the literature (human cancer cell derived xenografts grown in nude/SCID mice, receiving targeted radiotherapy) in the literature typically use 5-7 mice in each treatment group in order to achieve mean differences at 5% significance levels. However we will be comparing the efficacy of tumour growth inhibition by different radionuclides so the differences in growth rate are likely to be smaller so we may require larger treatment groups. We have sought advice from a statistician who has recommended a pilot study to assess the magnitude of cancer growth inhibition is achieved by the radioactive drugs to determine treatment group size.

PET is very sensitive and its quantitative accuracy is highly dependent on minimal subject motion which is best achieved through anaesthesia. Image degradation through movement would inevitably lead to the requirement of more animals per time point negating the benefit of reduction in animal numbers through serial imaging.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Pilot experiments will be used to check that the transplanted cells produce regular sized cancers suitable for autoradiography and that the radiation dose administered during treatment, determined from in vitro experiments and the literature, is sufficient to produce appreciable cancer growth inhibition compared with untreated tumours. We will seek advice from the University's biostatisticians regarding actual sample sizes required informed from pilot data.

Cell lines are authenticated and regularly tested for mycoplasma contamination by the University's core facilities.

(Reference: Workman et al Guidelines for the welfare and use of animals in cancer research. Br. J. cancer 2010 102:1555-1577)

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice used in these studies are inbred which will reduce genetic variability between the mice.
As described above we will use pilot studies to determine the growth inhibitory effect of targeted radiotherapy on xenografts to inform on the actual number of mice needed in the control and treatment groups.

Where possible experiments will be designed so that multiple treatment groups can be compared with a single control group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The procedures carried out in this study are the induction of carcinogenesis by subcutaneous injection of cancer cells into the flanks of nude mice followed some days later by injection of a radioactive agent that targets the cancer cells. These tumours do not usually spread. Tumour growth of sub-cutaneous tumours does not result in constriction of blood vessels or organs.

Why can’t you use animals that are less sentient?

Rodents are the animal of lowest sentience that can support the growth of xenografts and that can receive radiotherapy, hyperthermia and radioactive drugs. Nude mice are genetically altered so that they can be hosts to tumour derived from human cancers.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The generation of cancers in nude mice under anaesthetic will be painless. Once recovered from anaesthesia (within 1h) the mice feed and behave normally and do not display signs of discomfort. As the cancers progress the mice will be carefully monitored for behaviour suggesting discomfort. Generally the cancer enlarges with no apparent evidence of discomfort. These types of cancers (xenografts) do not usually spread beyond where the cancer cells were inserted. Occasionally (<10%) animals bearing xenografts display weight loss. Animals are weighed 2 times per week from the time the cancer cells were implanted. Once palpable the cancers are measured twice per week. From 0.75cm3 in volume (size of a garden pea) the tumours are measured daily and the animal weighed. Weight will be monitored and the mice will undergo euthanasia if their weight loss exceeds 15% of body weight from start of treatment (after subtracting an estimate of cancer weight).

PET is very sensitive and its quantitative accuracy is highly dependent on minimal subject motion which is best achieved through anaesthesia. Image degradation through movement would inevitably lead to the requirement of more animals per time point negating the benefit of reduction in animal numbers through serial imaging.
What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Workman et al Guidelines for the welfare of animals in cancer research. Br. J. Cancer 2010 102 1555-1577

An updated version of this paper is in progress. Recommendations in the revised version on publication will be followed.

NC3Rs website

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

NC3Rs website to follow any developments in cancer models. If a suitable cancer model becomes available which enables 3D tumours to be generated that are several mm in diameter in vitro we will compare results from dose-distribution experiments with that from xenografts in a cell line to determine if some of the work can be carried out using the model.

200. Quantitative metabolomics for prediction of lameness and elucidation of related mechanistic pathways in dairy cattle

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Dairy cattle, Lameness, Biomarkers, Metabolomics, Machine learning

Animal types	Life stages
Cattle	adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop methods to predict the risk of lameness (impaired mobility) and lesions causing lameness in dairy cattle using a combination of cutting-edge chemical analytical techniques and artificial intelligence.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Lameness (impaired mobility) is one of the highest priority diseases for the UK dairy industry. This painful and debilitating condition affects cattle welfare as well as production and health. Current estimates suggest that at any one time ~30% of dairy cows in UK herds are suffering from lameness. Implications extend beyond the cow to the sustainability of dairy farming and environmental impacts from disease reducing efficiencies. As a complex disease with many factors contributing to its occurrence, lameness is inherently challenging to tackle and major gaps exist in our understanding of disease processes contributing to lameness because of these challenges. Additionally there is currently a lack of data relating to the interplay between the lesions that cause lameness and the cow displaying impaired mobility (clinical lameness) due to resource challenges in collecting this data from appropriate sample sizes of cattle. Detection of lameness is currently only possible at an advanced stage of disease when pain causes cows to walk with an altered gait. The ability to detect lameness early on, prior to this stage, would be a huge advancement on the current situation resulting in improved health, welfare and productivity.

This work will generate data that will provide a vital insight into the disease pathways contributing to lameness in dairy cows and the ability to predict disease risk. This in turn will inform disease management and provide the building blocks to develop tools for early prediction of lameness, offering a step change in the detection and management of lameness in dairy cows. Additionally, the methods developed will be applicable to many other complex disease in both livestock and humans and will be further utilised in future work.

What outputs do you think you will see at the end of this project?

This research will develop methods to predict lameness risk during first lactation and identify key information from chemical analytics that show whether a cow is more likely to experience lameness. In turn, this information will be used to understand the biological pathways that contribute to lameness and lesions causing lameness. This work will advance knowledge of the fundamental biology of the disease pathways leading to lameness, enabling improvements in management strategies to reduce lameness and therefore improve welfare through reduction of this painful condition. Additionally, the methods developed will provide the building blocks for future development of diagnostic and management tools for lameness in dairy cows. For example, in-line milking diagnostic tools will enable individual cows that are more likely to experience lameness to be identified during routine milking and management decisions to be made (including breeding decisions). Findings of this work will be published in a number of peer-review publications. Data generated under this project will be presented at multiple national and international conferences by the principal investigator, post-doctoral researchers and PhD students.

In addition this project will result in the establishment of a bank of samples (biofluids) from two large groups of dairy cows entering their first lactation that will provide a valuable resource for future research studies and aid in the generation of further data for subsequent grant applications. Through future work involving a range of different chemical analytical techniques, analyses of these samples, utilising a range of non-animal techniques using artificial intelligence the research approaches can be developed reducing further need for animal research.

Who or what will benefit from these outputs, and how?

The research outputs have the potential to be extremely valuable to the dairy industry; providing the knowledge and foundation to construct a robust monitoring and preventive strategy for lameness. There will be direct and substantial benefits to animal welfare. Key beneficiaries include, animals, dairy farmers and consumers through improved understanding of the pathogenesis of lameness and the ability to develop tools to prevent and manage lameness, ultimately reducing the prevalence of a condition with major health, welfare and economic impacts. Societal benefits will come from improved sustainability through reduction of lameness in the national dairy herd of approximately 1.8 million dairy cows.

Outputs from the research will be used by industry through the development of management and diagnostic tools to reduce lameness in dairy herds as well as the research community through the ongoing investigation and optimisation of lameness management and the application of the methods developed to other complex diseases of livestock and humans. The research is funded by BBSRC and aligns with the BBSRC research priority areas of: ‘Animal Health’ and ‘Sustainably enhancing agricultural production’ within the strategic priorities of ‘Agriculture and food security’ and ‘Bioscience for heath’, as well as ‘Data driven biology’ under the enabling theme ‘Exploring New Ways of Working’ and ‘Welfare of managed animals’.

Establishment of a biobank will provide a valuable resource with benefits to the research community for grant applications and research as well as the animals, farmers, advisors and consumers benefiting from outputs of this research. The biobank will be managed by the PI and research group with storage of samples at -80oC and comprehensive cataloguing of samples. Access to samples and accompanying data will be available to the research group as well as internal and external research groups where appropriate for collaboration.

Short-term benefits will be realised through timely communication of results directly to the industry (via AHDB Dairy (the dairy farmers levy board for Great Britain), BCVA and other industry organisations), the research community and policy makers (via national and international conferences and peer- reviewed publications). Short term benefits will improve cow welfare and health and improve farm sustainability (through reduced disease, improved long-term cow heath and productivity).

Longer-term benefits will be realised through future work to develop and commercialise tools to predict lameness. These decision-making support tools will benefit farmers, farm advisors and vets involved in the health management of dairy cows. The research will provide the building blocks to develop these tools and findings will be communicated and made available to other scientists and industry through publication in peer-reviewed journals and presentation at scientific conferences and meetings. In terms of financial benefits, reduction of lameness will provide farms with a substantial reduction in economic losses. Lameness has been demonstrated to impact on production parameters including culling rates, fertility and milk yield with indirect costs in addition to the direct costs associated with the treatment of lameness. Estimates of costs for the average lameness event are in the region of £330, which is likely to be conservative figure, based on calculations reported in 2009. For a 200-cow herd with an average of 25% the herd lame at any one time (current estimated average for UK herds is around 30%), the costs per year for lameness have been estimated at £40,000 per year.

Additional longer-term benefits will be realised through the application of the methods developed to other complex disease of livestock and humans. Dissemination of research

findings to the scientific community through conferences and peer-reviewed publication will enable this work to be used for application to a wide range of diseases.

How will you look to maximise the outputs of this work?

To maximise the output from this project data will be published in high-impact, open access journals. Data collected under this project will also be presented at international and national conferences, meetings and seminars. Additionally we will use established networks (e.g. AHDB Dairy, BCVA, Dairy Cattle Mobility Steering Group and other working groups) to disseminate research findings.

Where possible we will collaborate and share resources (samples, knowledge, technical skills) with other researchers within and outside of the University, to maximise the scope and impact of our research.

It is our anticipation that data generated under this project (both positive and negative) will be made available.

Species and numbers of animals expected to be used

- Cattle: 320

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will follow a standard population of dairy cows from one unit to monitor for naturally occurring lameness outcomes at the prevalence and severity experienced by the average UK dairy herd, with accompanying collection of samples for metabolomics analysis (chemical analytics). In order to identify biological markers (biomarkers) for lameness and lesions causing lameness in dairy cattle it is necessary to conduct the research in the same species managed for milk production. This project therefore uses this type of animal at this life stage (adult cows entering lactation). There are no other species appropriate for this research.

In addition the use of dairy cows maintains continuity with existing preliminary research enabling data to be generated to further understanding of the pathogenesis of lameness in dairy cattle and identify robust biological markers for accurate prediction of lameness risk.

Typically, what will be done to an animal used in your project?

Under this project we will be evaluating differences in the metabolic profile of lame and non-lame first lactation dairy cows to identify biological markers for lameness and lesions causing lameness prior to the onset of clinical lameness (impaired gait).

Two separate cohorts of 160 cows (320 in total) will be housed and managed (including feeding, milking, disease and health management) according to the normal farm management routines for the rest of the herd in high quality, spacious facilities. At enrolment (~2-3 weeks pre-calving) urine and blood samples will be collected under restraint (minimum 1 week between sampling). From ~2 weeks post-calving blood, urine

and milk samples will be collected typically every 2 weeks (minimum 1 week between sampling) for either one lactation (about 305 days) or until the first lameness event (whichever occurs first). At the onset of lameness an appropriate intervention according to best practice will be made to treat the cause of lameness and cows will be removed from the study to remain within the herd at the existing farm facility. The definition of lameness will be based on a published 6 point mobility score scale (0, 1, 2a, 2b, 3a, 3b). Score 2a represents uncertain lameness and therefore two consecutive weeks at 2a will be used to define lameness. Sample collection will include; blood via a superficial vein (tail or jugular), hand-stripping of one quarter for milk and collection of urine via free-flow or catheterisation. Samples will be stored for later analysis using mass- spectrometry (chemical analytics).

Data relating to lameness (mobility score and foot lesion score) and body condition score will be recorded by visual assessment of cows in their normal housed environment. Lesion scoring will be conducted by lifting the feet in a cattle crush to inspect the claw surfaces and surrounding skin.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect a majority of the samples to be collected whilst cows are housed in their normal environment using normal husbandry methods of restraint (locking head yokes or stalls). Where it is more refined from a welfare perspective (e.g. collection of blood samples from an alternative site such as the jugular instead of the tail vein) or for a safety reason cows may be restrained in a cattle crush. Collection of blood samples from a superficial vein (tail or jugular) for metabolomics analysis may 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. Some cows may be catheterised for urine sample collection where it is not possible to collect urine via free-flow. This may result in minor discomfort and irritation at the site of sampling however the impact will be localised to the site of catheterisation and the duration should typically be no more than 12 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Over the duration of this programme of work, it is anticipated that 100% of animals will experience a procedure of a mild severity as blood samples will be collected from all cows on the study.

What will happen to animals at the end of this project?

- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Lameness is a complex condition commonly seen in dairy cows and aspects of the disease pathways of lesions causing lameness are specific to this species and risk factors relate to the way that dairy cows are managed. This project aims to identify biological markers for disease by investigating how differences in the metabolic profile (metabolome) relates to phenotypic state of lameness. It is therefore necessary to conduct this research in animals in order to achieve the aims of the project.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are not possible for this study type.

Why were they not suitable?

Expression of disease outcomes (lameness) is required in order to evaluate differences in the metabolome.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used in this project is based on pilot data which has demonstrated that for the sample size and methods being used (metabolomics and subsequent analysis of data generated using machine learning) the expected accuracy of prediction of lameness risk is >90%.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We conducted 2 separate pilot studies from 2 cohorts of dairy cows at the same unit to ensure the appropriate minimum sample size was identified. Robust analysis of these pilot data enabled the the accurate estimation of the minimum number of animals needed to maximise the likelihood of obtaining highly predictive algorithms; this number is used in the current study design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
Pilot studies have been conducted prior to the design of this project. Additionally this study involves the establishment of a biobank of samples that will be stored and catalogued for future use to ensure the samples collected will be easily accessible for ongoing research. Samples will be made available for under-and post-graduate studies within the University, 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.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Dairy cows represent the most appropriate animal model for studying lameness in this species and will enable the underlying biological mechanisms that are relevant to cows producing milk in a commercial farm setting to be studied in detail. Holstein cows have been chosen as this breed makes up the majority of milk producing cows in the UK so the results of this study will be applicable to most animals in the UK. Throughout the work, animals will be group-housed to provide near-commercial environments and conditions to ensure findings are relevant to the UK dairy industry. As such, our choice of animals and approach is the most refined for the intended purpose and the only possible method to meet our research aims. Their use also maintains continuity with existing data collected as part of pilot studies, therefore expanding on the data available to achieve the aims of the study.

Why can’t you use animals that are less sentient?

Lameness is primarily a disease of adult cattle and the underlying mechanisms being studied are likely specific to dairy cattle, therefore this stage of the species needs to be used to achieve the study aims and to make the substantial improvements in lameness urgently needed for the global population of dairy cattle.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals on the study will be monitored daily by farm staff and weekly monitoring of lameness and body condition score will be conducted as part of the research protocol. This level of monitoring will ensure any changes to condition, demeanour or behaviour or significant deviations from normal will be recorded, and where appropriate, result in discussion with the staff within the facility, the NVS or their deputy and appropriate action taken. Health and production parameters are also measured with a high frequency via the robotic milking system which alerts farm staff to health and production deviations from normal. These alerts will also result in the appropriate discussions. All animals will be sampled within their normal environment to minimise any welfare costs due to separation from the herd.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Throughout the course of this project we will make reference to multiple sources for best practice guidance including:

NC3R’s website for details on experimental design: https://nc3rs.org.uk/experimental-design

NC3R's ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular liaison with local NACWO and NVS. Subscription to the NC3Rs monthly email alerts provide the most recent developments in NC3Rs publications, guidance and information. We also receive regular updates and latest animal welfare guidance information via bulletins disseminated from our Biomedical Research unit.

201. Mechanisms regulating gene expression 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

gene expression, development, enhancer, transcription, RNA

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how genes are switched on and off in the correct cells, at the right time and at appropriate levels during the development of embryos and organs, and why inheriting mutations that affect this process can cause developmental disorders in humans.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important as it will allow us to:

test how cells switch genes on and off in cell types that are typically difficult to access in human patients

test if mutations that have been identified in patients with certain developmental disorders are responsible for that disease understand better how mutations in our DNA affect genes switching on and off so that we can better predict disease-causing mutations in patients and families

What outputs do you think you will see at the end of this project?

The primary outputs of this project will be research publications that improve our understanding of how genes work and how inherited mutations cause genetic disease.

Who or what will benefit from these outputs, and how?

Short-term impacts are likely to be primarily on scientific researchers working in these immediate research areas, and these impacts are likely to be continuous throughout the life of the project.

Medium-to-long term impacts beyond the timeframe of this project include better predictions of whether some types of inherited mutations will or will not cause developmental disorders, which has potential to impact on patients with these disorders and their families.

Longer term impacts might potentially extend to better understanding of how some developmental disorders arise, and the development of therapeutic approaches to treat or prevent these disorders.

How will you look to maximise the outputs of this work?

Outputs will be maximised through publication in high profile research journals, and publicity through conventional and social media channels. Work covered by previous versions of this project licence has been incorporated into popular science books and media.

Raw and processed data will be made available on public databases to allow other researchers to study and build on this work, and to identify any links that we might not have been able to find ourselves, potentially using approaches or techniques developed subsequently.

Genetically altered lines developed in this project will be made available to other researchers, and genetically altered lines that are likely to be in high demand will be deposited in appropriate repositories (e.g. MRC Harwell hub of the European Mouse Mutant Archive).

Dissemination of new knowledge that has potential to be translatable to the clinic will be achieved through collaboration with clinical colleagues.

Species and numbers of animals expected to be used

- Mice: 20000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse is a good model to study regulation of gene expression, its impact on normal development and how it can go wrong in human disease as there is a large volume of genetic and molecular knowledge in this area in this species. The work on exemplar disease loci such as *Sox9*, *Pax6* and *Shh* build on extensive detailed previous studies defining enhancer elements and gene regulatory mechanisms at these loci in mice. The availability of reagents such as antibodies, and existing genetically altered lines, that are used to study these processes also facilitate research using mouse models. The ease of genetic manipulation of this species, and the relatively short generation time are also advantageous.

This work will use mice containing genetic alterations in genes that affect regulation of gene expression. These animals will allow us to uncover and understand how gene expression is regulated during normal development, and how inherited genetic mutations can disrupt this process and cause developmental disorders. Non-genetically altered animals will also be used as controls, and to assess regulation of gene expression during normal development.

The choice of life-stages used is mainly determined by the gene expression patterns of the genes under study, the developmental timings of the organs in which these genes function, and the stages of development at which the associated developmental disorders arise. Most of the work in this project will be done on embryos, sometimes at developmental stages before the embryo becomes protected. Some work may be done in post-partum pups or in adults to assess longer term consequences of altered gene expression, or to study development of cells and organs where significant parts of their development occur during post-natal stages (e.g. neurons).

Typically, what will be done to an animal used in your project?

Most (>90%) of the animals used in this project will be used in breeding and maintenance procedures to generate mice with the desired genetic modification. Animals will typically be killed using a schedule 1 method, then post-mortem tissue dissected for subsequent analysis by molecular techniques.

Some mice (~5%) will experience an additional procedural intervention prior to killing and tissue collection e.g. intraperitoneal injection with the nucleotide analog BrdU to label proliferating cells.

A small number (~1%) of mice may experience ageing to 12 - 24 months to allow effects on age- dependent effects on regulation of gene expression to be studied (e.g. analysis of NMD reporter mice).

What are the expected impacts and/or adverse effects for the animals during your project?

The majority (>80%) of the mice used in this project are expected to experience sub-threshold levels of severity.
Some mice (~10%) used in this project are expected to experience mild levels of severity due to the nature of the genetic alteration used (e.g. mild growth restriction that does not impact on the ability of the animals to feed or drink, or detectably alter their movement or behaviour).

Some mice (~5%) used in this project are expected to experience mild levels of severity due to administration of agents (e.g. procedural intervention to intraperitoneally inject the nucleotide analog BrdU to label proliferating cells will result in transient mild threshold pain due to the injection itself).

A small number of animals (~1%) may also experience mild adverse effects associated with ageing to 12 - 24 months.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority (>80%) of the mice used in this protocol are expected to experience sub-threshold levels of severity.

Some mice (~20%) used in this protocol are expected to experience mild levels of severity due to the nature of the genetic alteration used (e.g. mild growth restriction), or due to administration of agents (e.g. procedural intervention to intraperitoneally inject the nucleotide analog BrdU to label proliferating cells), or due to ageing to 12 - 24 months.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The genes and mutations that will be studied in this project are associated with regulation of gene expression and originate from either genetic changes associated with developmental disorders, or from studies in cell line, biochemical or other animal models.

For genes and mutations that are associated with clinical mutations and human populations, animals are needed to study how these genes and mutations cause these developmental disorders as this type of mutation as relevant human tissue at the appropriate stage in development is typically difficult to obtain from normally developing embryos, and extremely difficult to obtain from embryos affected with these disorders. Therefore, animal models allow the effects of candidate genetic mutations and

polymorphisms to be studied in the relevant tissue and at the correct developmental stage to help understand how these mutations and polymorphisms cause to these disorders.

For genes and mutations that are associated that have already been studied in cell line models, biochemical or other animal models, animal use is often needed to test whether key aspects of gene function that have been uncovered in these other models is relevant to cells and tissues during normal development, and how these relate to developmental disorders. Therefore animal models are needed to study mechanistic aspects of gene expression regulation in transient and dynamic developing tissues that are often not particularly well modelled in cell culture, or readily available from human embryos.

Which non-animal alternatives did you consider for use in this project?

Cell-free *in vitro* models, computational predictions and cell line models were, and will continue to be considered during the course of this project. Notably, some of the mechanisms that will be studied in this project build on information obtained from work from some of these alternative systems.

This project is part of a larger programme of work that incorporates biology across scales, spanning computational predictive models, cell line models, animal models and human population genetics and human clinical genetics. The animal use within this project is typically a component of a larger piece of work spanning at least two of these experimental approaches, and will model and validate key findings from *in vitro* experiments and clinical data.

Why were they not suitable?

Biochemical reconstitution of gene expression in its entirety is not currently possible, therefore could not be used to replace animal models in this project. Biochemical reconstitution of individual reactions within this process can provide some insight but likely still lack key components and are restricted in scope.

Cell line models are the main alternative to developing embryonic cells and tissues, however cell lines typically do not model these transient and dynamic developing tissues particularly well. Many of the genes that will be studied in this project have been studied in cell line models that approximate some aspects of development (e.g. embryonic stem cells, differentiating embryonic stem cells), but key findings need to be confirmed in the tissues in which these genes are normally expressed and developmental orders typically arise.

For studies involving regulatory elements such as enhancers: these modular regulatory elements are typically tissue-specific and active only transiently during development, with different cell types and developmental stages using distinct enhancers. Therefore some experiments relating to these elements need to be done in the developing tissues where these elements are normally active.

Computational predictions of the effects of non-coding mutations on gene expression are still developing, and are not at the same level of accuracy as those involved in predicting the effects of coding mutations on protein activity. Therefore these methods are currently not reliable to predict the impact of many non-coding mutations and genetic variation on disease. The work that will be undertaken in this project will improve our understanding of how non-coding mutations and genetic variation impact on gene expression.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on similar projects currently undertaken on three expiring project licences held at MRC Human Genetics Unit that have this overlapping research theme (P93007F29, P2A477A62, and P76777883).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The majority of the animals used in this project are used in breeding and maintenance protocols to generate experimental animals of the desired sex and genotype.

I have consulted with a statistician to determine appropriate sample sizes and experimental design to associate genotype with phenotypic effects in these cohorts of experimental and control animals. Data from previous phenotypic analyses were used to generate estimates of data distributions and effect sizes to facilitate those sample size calculations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Cell line models, structural predictions, and biochemical assays will be used where possible to design and test specific types of genetic alteration, or to test functionality of fusion proteins, to identify the key genetic alterations to introduce into mice. This will reduce the number of mice used by limiting the genetic alterations studied to those that are likely to be most informative.

Efficient breeding strategies will be used to limit the number of animals used. Genetically altered lines will be cryopreserved to facilitate sharing and distribution of these lines, and to limit breeding and maintenance once all experimental cohorts have been collected.

Multiple phenotypic assays can typically be performed on tissue isolated from each animal, and some material frozen for use in multiple molecular assays over long time scales, reducing the number of animals needed to generate significant mechanistic insight.

For some aspects of this project, cell line models (e.g. neural stem cells) can be established from genetically altered lines that can allow extensive detailed molecular laboratory analysis that can continue for years.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of the animals used in this project (>70%) will experience breeding and maintenance protocols, but will not experience any additional procedural intervention, and will therefore experience minimal distress or harm. The genetic alterations typically involve genes and pathways involved in regulation of gene expression, and the pain and suffering associated with these genetic alterations will be minimised by using humane end-points that precede the onset of clinical symptoms. The genetically altered tissue that will be obtained from these animals will be obtained post-mortem after killing by a schedule 1 method.

Some animals will experience additional procedural interventions (e.g. injection of agents to activate/inactivate transgenes) prior to killing by a schedule 1 method and tissue collection. This is expected to cause mild and transient discomfort, but is not expected to cause lasting harm to the animals. These additional procedural interventions are often refinements that restrict any potential harmful effects of a transgene to specific tissues and times in relation to the injection, reducing the
harmful effects and severity of a genetic alteration (injection of agents to activate/inactivate transgenes).

Why can’t you use animals that are less sentient?

The life stages used for these experiments are largely dictated by developmental disorders that are being studied, and the expression patterns of the associated genes. Experiments that model developmental disorders caused by altered expression of developmentally regulated genes must be carried out at the embryonic stages during which these genes are expressed and the developmental disorders arise. Experiments that model developmental events that occur post-natally (e.g. neurons, spermatogenesis) will need to use post-natal mice.

Animals that are less sentient (e.g. nematode worms, fruit flies) often do not use the same genes, developmental mechanisms or have the relevant developmental tissues as some of the processes under investigation in this project. Zebrafish will be used to model some aspects of eye development and developmental gene regulation where appropriate under this programme of work, but this will be undertaken under a separate project licence.

Most of the animals used in this project (>80%) will experience breeding and maintenance protocols, but will not experience any additional procedural intervention. The genetically altered tissue that will be obtained from these animals will be obtained post-mortem after killing by a schedule 1 method, therefore in most cases terminal anaesthesia does not provide significant benefits. However, on occasions, terminal anaesthesia may be used to allow exsanguination, when we require to limit blood contamination of tissue for downstream studies or for perfusion or perfusion fixation when we require to ensure compete tissue architecture is retained or for organ/tissue removal when we require to remove organs immediately to snap freeze to maintain RNA integrity at the point of culling.

Occasionally, when we need to limit blood contamination of tissue for downstream studies by exsanguination, or to limit subsequent blood or immune cell contamination of tissue by perfusion, or to retain tissue architecture by perfusion fixation, or to maintain RNA integrity

at the point of culling by immediately snap freeze organs, terminally anaesthetised animals will be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of the animals (>70%) will only experience breeding and maintenance protocols. Refinements associated with this protocol include environmental enrichment, and implementation of non-aversive mouse handling.

Any necessary injections will be refined by implementation of non-aversive mouse handling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs - Responsibility in the use of animals in bioscience research
https://nc3rs.org.uk/3rs-resources/responsibility-use-animals-bioscience-research

Medical Research Council - Guidance on research proposals involving animal use
https://www.ukri.org/councils/mrc/guidance-for-applicants/proposals-involving-animal-use/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Annual PIL refresher courses run by the University will help keep me, and other PIL holders implementing this project, informed about advances in 3Rs.

Experimental interventions will be authorised locally by the our Named Veterinary Surgeons through a time-limited Experimental Request Form (maximum duration 1 year), which will allow timely implementation of 3R advances in these regulated procedures.

202. Recognition memory in an animal model of Alzheimer's disease: Associative mechanisms

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Association, Recognition memory, Alzheimer's disease, Mouse

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to refine understanding of the psychological mechanism underlying a specific recognition memory deficit we have found in a young transgenic mouse model of Alzheimer's disease (AD), and which we think could serve as a cognitive marker of preclinical AD.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The brain changes that characterise Alzheimer's disease (AD) begin many years before symptoms become apparent, and once the condition is diagnosed the condition can be difficult to treat because the brain damage is advanced. Thus early diagnosis and treatment of AD is a research priority, and this requires development of effective screening tools to identify AD at an early stage, as well as evaluation of prospective treatments. Psychological tests are ideal for screening purposes as they are cheap, noninvasive and easy to administer, and if such tests are translational, they can also be used for drug evaluation in preclinical studies. The specific aim of this work is to refine a translational

psychological test that our previous work has shown is indicative of early cognitive deficits in a mouse model of AD.

Our previous work on a transgenic model of AD identified a subtle and early-emerging pattern of deficits in one particular, well characterised mouse model of AD, the APPswe/PS1DE9. We used an object recognition task, performance on which relies on a rodent's natural curiosity. If mice explore an object and are then permitted to explore it and a second, novel object, they preferentially explore the novel object - suggesting they recognise the previously experienced one. We interpret this behaviour in terms of associative theory, which explains recognition in terms of two processes, self-priming and retrieval-priming. Self-priming refers to the phenomenon in which a stimulus is processed less well if it has been repeated; for example, if someone bangs on the door twice, you would less startled the second time).In contrast, retrieval-priming refers to the processing of a stimulus being reduced when it is anticipated (for example a ring on the doorbell might make you jump, but less so if you heard the footsteps coming up the path. We found that although performance on the standard recognition task was normal in these young mice, specially designed task variants that measure self-priming and retrieval-priming separately revealed a selective deficit in retrieval-priming in the transgenic mice, compared to wild-type littermates. This subtle deficit emerged at 4-5-months of age, a point at which neuropathology is only beginning to develop in this strain, and so could be an important diagnostic sign of preclinical AD.

We interpreted this finding in terms of an established model of associative learning, according to which these impairments reflect a deficit in an associative learning process. If this hypothesis is correct, it implies that performance should also be impaired on a wider range of tasks that depend on such associative learning. It also raises the question as to whether it is association formation that is impaired, or whether the deficit stems from e.g. a failure to process certain types of stimuli involved in an association (e.g., spatial cues), or is only evident when the to-be-associated events are separated in time or space. The work in Objective 1 aims to establish the boundary conditions under which this associative learning deficit may be observed, refining our characterisation of the deficit seen in these animals. This will be achieved partly by studies using the recognition-type task; however, an integral part of our hypothesis is that association formation might be impaired in these animals, and as association formation is time-sensitive, some of the experiments will require use of a Skinner-box type task, motivated by mild food deprivation, as these afford a degree of temporal control over the delivery of the various stimuli that is impossible in the recognition tasks. Some of these Skinner-box tasks will require pilot work in non-transgenic, C57 mice, to refine the parameters before the transgenic animals are put through the procedures.

This work is key to improving our understanding of the cognitive processes impaired in prodromal AD, and will inform future development of diagnostic tests suitable as screening tools, and translational tasks for testing new drug treatments.

This work has a more general importance. Recognition memory is a theoretically important phenomenon that is impaired in dementia and also normal aging, and recognition memory tasks are routinely used across the field of neuroscience. Our theoretical interpretation of this effect in terms of an associative mechanism is an advance over previous accounts, as it seeks to explain recognition in terms of a more general theory of learning. Thus, the proposed work will have significance beyond its specific application to Alzheimer's disease.

What outputs do you think you will see at the end of this project?

First, this work will give us new theoretical information about the nature of recognition memory impairments in the early stages of the disease in this transgenic model. This will, more generally, increase our understanding of how cognitive impairments develop in AD, which is of clear theoretical interest to those with a specific interest in AD. In addition, these experiments are motivated by application of a new, associative theory of recognition memory. Thus it will also be of immediate interest to those concerned with the theoretical associative mechanisms underlying recognition memory.

The publication outputs of this project primarily comprise scientific articles that will be published in leading journals, and presentation at international conferences.

Who or what will benefit from these outputs, and how?

The behavioural results will be of immediate benefit to those studying the early cognitive correlates of AD and also to those interested in the study of associative learning, and how it may be applied to broader phenomena like recognition memory. Also, as the work develops a translational model of recognition memory, it will be of interest to the many neuroscience researchers using tasks of this type to evaluate memory performance.

In the medium term, as this work will provide information about the potential recognition memory deficits that may be present in human patients in the early stages of AD, it will be of benefit to more clinically oriented researchers who are interested in developing new diagnostic tests for AD.

In the longer term this work could potentially result in a new, diagnostic test for AD, that could be used by clinical practitioners to identify people in the early stages of the disease who could benefit from potential treatments.

How will you look to maximise the outputs of this work?

The outputs will be maximised by rapid publication of the key findings, including null results, presentation at both national and international scientific conferences (e.g. Associative Learning Symposium UK, Associative Learning Group, Australia, Alzheimer's Research UK, British Neuroscience Association), and also more general dissemination public engagement events (e.g. Pint of Science) and also interested user groups (e.g. IMH dementia groups).

Species and numbers of animals expected to be used

- Mice: 1840

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Broadly, the aim of this project is to conduct a theoretical analysis of the subtle recognition memory deficits seen in this model, at an age equivalent to human prodromal AD. The work on which this project is based was conducted in mice, specifically the APPswe/PS1DE9 mouse, and so we propose to continue using this strain. This strain is

highly characterised, and there is a considerable body of relevant previous work using this specific model which justifies its continued use in this project.

Specifically:

previous research allows us to pinpoint the age equivalent to the prodromal stage of AD: systematic evaluation of the development of the amyloid plaques characteristic of AD in these animals has revealed that they first appear at 4-5 months of age, but are only consistently observed from 6 months (Garcia-Alloza et al., 2006). Other signs of neuropathology appear much earlier, however; Zhang et al. (2012) first observed elevated levels of oligomeric Aβ at 4 months of age in these mice, and these were positively correlated with indices of cholinergic dysfunction and oxidative damage – which the authors interpreted as early pathological responses to oligomeric Aβ. These authors also found that cognitive deficits appeared at the same age. This work underpins a key assumption of this project, that 4-5 months of age may be regarded as equivalent to the preclinical stage of AD.

an unusually high number of studies in this strain have examined performance on the spontaneous object recognition (SOR) test of recognition memory, on which this work is based. From this research a clear picture emerges: recognition deficits are routinely observed in older mice (9-12 months) but only occasionally reported at 6-7 months (e.g. Donkin et al., 2010; Jardanhazi-Kurutz et al., 2010; Li et al., 2014; Yan et al., 2009; Yoshiike et al., 2009; but see Frye & Walf, 2008), but never at less than 6 months (Bonardi et al., 2011; Bonardi, Pardon & Armstrong, 2016; Pedros et al., 2014). These findings underlie the second key assumption underlying this project, that performance on the simple object recognition task is not impaired in the 4-5-month-old preclinical stage of the disease in this model, and only subtle deficits are evident at this point. Our own work has explored these postulated subtle recognition deficits in this strain. We argue that SOR recognition performance comprises the component processes of self-priming and retrieval-priming, and we have observed a selective deficit in the retrieval-priming process in 4-5-month old mice of this strain. This result underpins our main hypothesis, that components of object recognition ability are impaired at this specific, early stage.

Some of these studies will require preliminary pilot work to refine parameter choice, and these will be conducted on C57 mice of approximately the same age, as these are a base strain for APPswe/PS1DE9 animals.

Typically, what will be done to an animal used in your project?

The first type of behavioural study, a recognition-type task, might involve:

extensive habituation to the apparatus

a series of recognition-type tests to evaluate associative interpretation of recognition memory performance. All behavioural measures are taken by videoing the animals with cameras placed above the arenas.

Habituation involves a series of placements, typically six of 10-min duration, in which the animal is placed in the experimental arena (a white perspex box with high walls) and allowed to explore. After this period it is returned to the home cage.
This behavioural recognition-type test typically comprises a series of occasions (2-4) in which the mouse is placed in the arena for a short period of time (10-15 minutes). These placements may occur within a day or across days, with interplacement intervals ranging

from 5 minutes to 24 hours. During these placements a number (2-4) of small, domestic junk objects (e.g. salt cellars, ashtrays etc) are placed in the arena, which the animals are allowed to explore. The arenas may also be lined with various inert materials to make aspects of this surrounding 'context' more distinctive; these materials could include covering the walls with patterned linings, or the floors with distinctively textured materials.

Animals might experience 4-6 such behavioural tests in a typical study. The entire sequence of tests (including rest days) would normally be no more than 4 weeks.

A second type of behavioural study, a Skinner box task, would involve

mild food deprivation (gradual reduction to no less than 80% of free feeding weight – i.e. no more than 20% weight loss relative to free feeding weight – over the course of about a week), and then this weight level is maintained over the course of the study by controlled feeding. This level of deprivation is usually considered mild.

a series of testing sessions in a Skinner box, in which the animals are trained that various combinations of motivationally neutral stimuli (e.g. low intensity auditory or visual stimuli) are either paired or not paired with outcomes such as food or sucrose pellets. Testing sessions would be typically conducted once daily, and each session would last for about an hour. An animals would typically experience no more than one Skinner box-type task, and a typical experiment would last about 2-3 weeks.

An animal would experience either mild food deprivation and a Skinner box-type task, or one or more recognition-type tests, or both types of task.

If recognition testing follows a Skinner box-type study, it would be preceded by a period of at least three days of access to full food. The total duration of testing for an animal would normally not exceed 7 weeks

What are the expected impacts and/or adverse effects for the animals during your project?

The recognition-type task involves placing animals into an arena and allowing them to explore small, domestic junk objects (e.g. salt cellars, ashtrays etc) while they are video-recorded. The only adverse effect we have seen is mild stress on initial placements - evident as a tendency to freeze and defecate. Both are typically confined to the first time the mouse is in the arena. Thereafter the mice seem relaxed – which is critical to the success of these experiments, which rely on the mice being willing to freely explore the objects presented.

The Skinner-box type task involves mild food deprivation; no specific adverse effects associated with food deprivation have been reported with this strain, nor major differences in growth rate compared to control mice. Placing the animals into the Skinner box chambers will initially be mildly stressful, but this quickly dissipates, being typically confined to the first time the mouse is in the chamber. That the mice are relaxed during training is critical to the success of these experiments, which rely on them being able to learn efficiently.
Perhaps the main potential adverse effect of this project stems from the use of this strain of transgenic mouse. In common with other strains with these mutations, it has an elevated mortality rate (10.5% for the colony as a whole, approximately 4% in our experiments). These deaths are sudden, and we have never yet experienced any sign that the animal suffers beforehand; for instance, in one case we have video footage of an animal

exhibiting normal exploration behaviour about an hour before it was found dead. Evidence suggests that these deaths are the likely the result of brief (~30-s duration) seizures that occur in this strain, which in a few cases can be fatal (Minkiviciene et al., 2009). Consistent with this interpretation, none of the deaths we have observed are associated with any post-mortem pathology. We have no evidence that mortality is elevated by the behavioural task. At the end of the experiments the mice will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Transgenic mice: Moderate; Pilot work in normal mice: Mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Achieving the objectives requires observation of behaviour, and so we must use live animals. Performing the studies in patients at an equivalent stage of the disease is impossible, as early diagnosis is impossible (developing an early diagnostic test being the motivator for the work).

Which non-animal alternatives did you consider for use in this project?

None - achieving the objectives requires observation of behaviour.

Why were they not suitable?

Because achieving the objectives requires observation of 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?

For the behavioural work our initial estimate of sample size for these studies is guided by power analysis of our previous work (using Gpower Erdfelder, E., Faul, F., & Buchner, A., 1996), which yields approximately 16 animals per group, and hence 32 per experiment. Assuming 10 experiments per year over 5 years, this yields 1600 animals in total. An

additional 3 experiments per year will also be required to pilot the Skinner box tasks; these will be within-subject experiments, and so add 16 x 3 = 48 animals per year, and an additional 240 animals over the course of the project. This gives a total of 1840 animals, 1600 transgenic and 240 C57 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will minimise the number of animals by using within-animal comparisons for our key recognition measures and analysing behaviour over repeated time periods. We will also use statistical techniques to estimate the minimum number of mice consistent with obtaining a scientifically meaningful result. We will reduce variability by counterbalancing the stimuli, objects, arena linings etc, and ensuring the animals are well habituated to the apparatus and extensively handled before the start of testing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Key effects will be routinely piloted in non-transgenic mice to perfect methodology, and hence optimise estimates of sample size.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Achieving the scientific objectives of this work, and so realising the benefit of this project, requires us to continue using the APPswe/PS1DE9 mouse model. This choice is driven by the fact that the assumptions underlying the proposed project stem from the considerable body of previous work using this specific model, and that the critical evidence on which this project is based comes from work with this strain. Although these animals develop a form of Alzheimer's disease, this does not affect their behaviour in any obvious way, aside from a slight tendency to be more active. This strain does, as is common with genetically manipulated animals, have a slightly elevated mortality rate (4% in our experiments), but these deaths are sudden and unpredictable, and not preceded by any sign of distress or accompanied by any obvious pathology**;** evidence suggests they are caused by brief (~30s) seizures that are characteristic in this strain (Minkeviciene et al., 2009).

The behavioural experiments we propose are very benign. The recognition-type tasks involve placing the mice in an arena where they are free to explore small domestic objects, such as ashtrays and salt cellars – which could be regarded as a form of environmental enrichment. The Skinner box-type tasks involve placement in a Skinner-box chamber and presentation of pairings of various combinations of benign stimuli (e.g. low intensity tones, lights etc) and food or sucrose pellets. It is important for the quality of the results obtained to ensure the mice are relaxed enough in this apparatus to explore the objects, and so

every effort is made to minimise stress in these tasks by extensive habituation to the apparatus and handling of the animals before the start of testing.

The mild food deprivation required for the Skinner-box type tasks will be at the minimum level compatible with obtaining reliable experimental results, and never less than 80% of growth-adjusted free-feeding weight. In fact restricted feeding can increase longevity in rodents: life expectancy of laboratory rats maintained at 80% ad lib weight is longer than that for rats maintained on ad lib feeding (e.g. Vitousek, Gray & Grubbs, 2004).

Why can’t you use animals that are less sentient?

Because the work relies on behavioural measures, which requires use of an awake and moving animal, and because the age at which the experiments are conducted is central to our experimental hypothesis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Optimal performance on these behavioural tasks requires the animals to be relaxed enough to explore the objects or learn the Skinner-box tasks, and this is an additional motivation to constantly keep an eye on refining our procedures. The extended habituation procedure is one example of this; another is the introduction of daily handling a number of days before the behavioural testing begins. Moreover, although as noted the elevated mortality in the transgenic mice does not have an obvious cause or trigger, in the eventuality that it is exacerbated by stress these refinements should be of benefit in this respect also. The food deprivation schedules will be the minimum required to obtain reliable performance, and this will be monitored throughout.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult resources such as N3CR and LASA websites and newsletters (see above) on an ongoing basis, as well as routinely using ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We subscribe to the regular NC3Rs newsletter, ensuring we are kept informed about all advances in refinement, so that we can implement all relevant measures promptly.

203. Defining early entry mechanisms of Mycobacterium spp. into the host

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

Mycobacteria, Pathogenesis, Diagnosis, Cattle

Animal types	Life stages
Cattle	juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Johne's disease (JD) is a common and chronic disease of the gut of ruminants caused by infection with the pathogen Mycobacterium avium subspecies paratuberculosis (MAP). The aim of this project is to investigate pathogen-encoded factors which allow MAP to invade the gut. By better understanding the pathogen-host interactions at the early stages of infection, we hope to identify cellular pathways that could be targeted to facilitate the rational design of strategies to control and treat MAP 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.

Why is it important to undertake this work?

JD has a significant impact on the health and welfare of cattle leading to reduced productivity, economic losses and, ultimately, death. At early stages following infection animals show little evidence of the disease, making it difficult to detect and easy for infection to spread unnoticed. Currently, reliable effective vaccines and treatments are not

available for JD so control programmes rely on detect and cull policies which are ineffective and expensive. A better understanding of the biology of JD, particularly of pathogenesis and the complex host-pathogen interactions in early MAP infection, is required to develop improved diagnostic tools and novel vaccines.

What outputs do you think you will see at the end of this project?

Outputs arising from this project include:

Determination of the cell types involved in transit of MAP across the gut lining to accurately identify the cellular portal of entry

Characterisation of the mechanisms by which MAP crosses the gut lining to identify critical cellular pathways and mechanisms of entry

Identification of critical MAP genes required for host tissue invasion by comparing e.g. naturally occurring field strains of MAP versus mutant strains

Who or what will benefit from these outputs, and how?

The principal beneficiaries of the studies conducted under this Project are:

Researchers studying JD, MAP and other intestinal diseases who will benefit from data on early events following MAP infection including key cellular responses and changes in bacterial function and gene expression

Students, research assistants, post-doctoral and early career scientists will benefit from training opportunities in diverse areas including molecular biology, cellular and molecular immunology, pathology and in vivo research with large animals

The pharmaceutical industry will benefit since we will pinpoint areas for the development of new diagnostics, treatments and vaccines

Policy makers, the farming industry and the dairy industry will benefit from data which has the potential to improve JD diagnosis and management strategies, and reduce economic losses associated with JD.

Animals affected by JD will benefit from improved JD diagnosis and management resulting in reduced disease occurrence and reduced suffering of individual animals.

Work arising from this Project will be of interest to the general public, not least because of the publicised but controversial link between MAP and Crohn's disease in humans, affecting approximately 1 in 1,000 people in the UK. Ultimately a reduction in JD will result in lower levels of human exposure to MAP in animal products.

How will you look to maximise the outputs of this work?

Subject to approval from academic partners, we will endeavour to publish as much information as possible from this Project:

We will engage with policy makers and academics through publications, presentations at conferences, invited lectures, via our website pages and through links with relevant professional societies.

We will identify, protect and exploit intellectual property and engage with industry as appropriate
We will exploit existing links to those delivering knowledge and tools to the farming industry and will engage with veterinary surgeons responsible for delivery of JD diagnosis and management strategies.

We will use short films, public presentations and lay articles to engage with the general public including promoting education in schools through a series of events organised through the host institutions.

Species and numbers of animals expected to be used

- Cattle: 45

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 calves <5 months of age since this is representative of the age at which calves are typically exposed to MAP on farms.

Typically, what will be done to an animal used in your project?

Typically the following will be done to animals in this project:

For 24 hours prior to surgery food will be withheld but animals allowed free access to water.

Animals will be anaesthetised and maintained under anaesthetic throughout the procedure which will be of 2-18 hours duration.

Surgical procedure - The peritoneal cavity will be opened and loops will be made in the small intestine by ligation. An inoculum of infectious agent will be injected into each loop, the loops replaced into the abdomen, and the abdomen closed. At various time-points post-challenge the abdomen will be re-opened and the intestinal loops will be excised and removed. Following removal of the loops between 2 and 18 hours post-challenge animals will be humanely killed by an approved method while still anaesthetised.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect to see any adverse effect in the procedures carried out under this Project.
Animals will be anaesthetised prior to any procedures being performed and will be maintained under anaesthetic throughout the surgery and should therefore not experience any adverse effects other than an intravenous injection to induce anaesthesia.

Animals will be killed by an approved method while still 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 animal type)?

100% of calves will experience a Non-recovery severity limit

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of a gut-loop model of infection allows detailed investigation of the initial stages of Mycobacterium avium subsp. paratuberculosis (MAP) infection at the relevant areas of the gut, which could not be achieved using cell-based systems or standard oral challenge of animals.

Which non-animal alternatives did you consider for use in this project?

We considered a number of in vitro models including an M-cell like cell culture system, MAC-T epithelial cell lines, polarised epithelial cell lines, and intestinal organoids. However, none of these models are able to accurately recreate the complexity of pathogen-host interactions of MAP at the gut lining.

Why were they not suitable?

All of in vitro models we considered are unable to accurately recreate the complexity of the gut lining and MAP pathogen-host interactions to a degree that is biologically relevant.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated 9 animals per year includes up to 7 animals that will undergo the gut loop procedure as well as up to 2 additional animals to be made available in case of anaesthetic death or surgical complications.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals used in this Project has been scrutinised and approved by an internal Experiments and Ethical Review Committee in conjunction with statistical advice to ensure that as few animals as possible are used to generate statistically 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?

In each animal, multiple gut loops will be created in multiple regions of the gut. For each gut loop section, tissues collected will be subject to multiple different tests. By maximising the number of gut loops created in a single animal and the number of tests performed on samples from each gut section we will minimise the total number of animals required while still generating statistically robust data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Calves <5 weeks old will be use since this represents the age at which calves are exposed to MAP on farm. Calves will be group housed prior to entering the Project to minimise distress.

Procedures will be performed under general anaesthesia and animals' vital signs will be monitored closely throughout. Animals will be humanely killed by a Schedule 1 method while still under anaesthesia (non-recovery). Since animals are not conscious during the procedure they will not experience pain or distress.

Why can’t you use animals that are less sentient?

We strive to use the least sentient animal possible whilst still maintaining the objectives of the Project. Calves <5 weeks old will be use since this represents the age at which calves are exposed to MAP on farm. It is not biologically relevant to use a more immature life stage.

The procedure will be performed under terminal anaesthesia so that the animals are not conscious at any point during the procedure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For the duration of anaesthesia animals' vital signs will be monitored. Additional supportive measures e.g. intravenous fluids, heating pads etc. will be available if required to keep the animals as comfortable as possible during the procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are no specific best practice guidelines for the specific procedure used in this Project. More general best practice guidelines including NC3Rs, ARRIVE, PREPARE and RSPCA resources will be consulted and followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of my institution's 3Rs Committee which meets on a monthly basis to discuss and advise on advances in this area. There are a number of 3Rs Committee members who are also on my institution's Animal Welfare and Ethics Review Board (AWERB), and who will be able to advise on effective implementation of any 3Rs advances that arise during the project.

204. Investigating mechanisms, novel therapeutics and diagnostics in thrombus formation and its resolution

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Thrombosis (clots), Inflammation, Stents, Atherosclerosis, Imaging

Animal types	Life stages
Mice	adult, pregnant, neonate, juvenile, embryo, aged
Rats	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The objectives of this project are to address questions in 2 main interconnecting areas.

Molecular and cellular mechanisms - What are the molecular and cellular interactions that: (i) give rise to a blood clot (thrombus) in blood vessels; (ii) regulate the natural removal of a thrombus by the body (natural thrombus resolution); (iii) give rise to a thrombus that forms inside wire mesh devices (stents) deployed to restore blood flow in vessels with existing clots; and (iv) are responsible for the effects of thrombosis on the development of other inflammatory vascular conditions such as the build up of fatty deposits in the arteries (atherosclerosis)?

Treatments and diagnostics - Can we use information gleaned from our mechanistic studies to: (i) target novel pathways that prevent thrombus formation, enhance the resolution of an existing thrombus, or prevent the potentiating effect of thrombosis on the

development of atherosclerosis in arteries; (ii) identify biomarkers that can be quantified to provide accurate and specific measures of the presence and structure of a thrombus; (iii) further develop and refine imaging methods (e.g MRI) to provide objective information on thrombus size and structure and hence susceptability to being dissolved by thrombolytic agents (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?

Thrombosis in blood vessels (arteries and veins) that help move blood around the body (to and from major organs, such as the heart and brain), are a major cause of death and long-term conditions including paralysis (from strokes), leg ulceration and limb loss, which are debilitating and inflict a considerable economic burden on healthcare provision.

Current treatments include: the use of drugs (anticoagulants) that stop further thrombus development (allowing their natural removal by the body); enzymes (thrombolytics) that break up clots; or interventions that either surgically removes the thrombus, or deploy a wire mesh device (stent) to re- open a vessel by pushing the thrombus against the vessel wall. The problems with these treatments are that they can lead to a significant risk of fatal bleeding, or re-thrombosis in up to 30% of stented vessels that will then necessitate further intervention.

What outputs do you think you will see at the end of this project?

A better understanding of the mechanisms that regulate thrombus formation and its natural resolution, in particular the relationship between these processes and inflammation.

The development of novel therapeutics that prevent the formation of a thrombus or promote its dissolution without the risk of bleeding.

The development of novel therapeutics that enhance natural removal of already formed thrombus without the risk of bleeding.

The development of imaging methods (e.g. MRI) that facilitate better targeting of patients for treatment e.g. show which clots can be removed by dissolving agents (lytics) and those that will require placement of devices (stents) that push aside the existing clot to establish blood flow in the affected blood vessel.

High impact publications that will disseminate our findings to the greater scientific and medical community and stimulate further work and clinical trials in this area.

Who or what will benefit from these outputs, and how?

Several of our findings could be readily translated into patients in the short term. For example, detecting thrombi that are dissolvable could reduce the incidence of heart attack, stroke and the development of chronic ulceration in the limb affected by thrombosis (post-thrombotic syndrome). In the longer term, our understanding of mechanistic processes that lead to cardiovascular disease discoveries of drugs that prevent thrombosis, or enhance

its removal, without risk of excessive bleeding would lead to improved outcomes in the clinic.

How will you look to maximise the outputs of this work?

We will closely collaborate with clinical colleagues to ensure that any therapeutic strategy or imaging method derived from our work can be tested in clinical trials for safety and efficacy and ultimately find widespread clinical use.

We will also disseminate the results of this work at national and international conferences and workshops and publish in peer reviewed international scientific journals in line with the ARRIVE guidelines (https://arriveguidelines.org/).

Workshops will be organised within our institution where we will share our results and provide hands on training for internal and external academics interested in this work. Other means of dissemination will include talks: at patient and carer events organised by the Vascular Society of Great Britain and Ireland and The Circulation Foundation; the Pint of Science initiative; and active participation in the summer festival of the Royal Society.

Species and numbers of animals expected to be used

- Mice: 7800
- Rats: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rodent models of thrombosis will be used in this project licence because their molecular and cellular responses to clotting and natural thrombus removal by the body are similar to those seen in patients with this condition.

Typically, what will be done to an animal used in your project?

Rodents used for the thrombosis models may undergo the following.

Bred to express certain gene defects that will allow us to interrogate the importance of those genes in thrombosis; or be given a diet that promotes a similar condition (fatty arteries) to that seen in patients who develop thrombosis in their arteries.

Undergo an operation to create a thrombosis in a vein or artery followed by treatment with agents, cells or bone marrow that might help prevent thrombosis or promote its resolution. These will be administered via direct injection into the thrombus; injection via a blood vessel, muscle, beneath the skin, or into the abdomen; or infused by a mini-pump placed inside the animal.

Imaging over time (by e.g. ultrasound or MRI) to determine the size and structure of the thrombus.

Have small blood samples taken at the time of imaging to evaluate the body's cellular and molecular responses to the thrombus.
Humanely killed and the vessel containing the thrombus, together with a blood sample, removed for analysis in the laboratory.

The duration of this experiment will typically be up to 28days. This will depend on whether the question be asked relates to processes involved in thrombus formation or its natural resolution.

Rodents used as a cell donors may undergo the following.

Bred to express specific gene defects so that cells obtained from these animals also express the same gene defect.

Administration of an agent to induce production of cells we believe may be important in thrombus removal (e.g. specific subset of inflammatory cells) via: (i) injection of the agent into their abdomen (peritoneum) to promote accumulation of the required cells in the peritoneum; or (ii) implantation of a mini-pump placed surgically into the abdomen to deliver the agent over a period of time in order to promote the release of cells from the bone marrow.

Humanely killed and cells removed from the abdomen, blood, or bone marrow for use in our mouse models of thrombosis or further analysis in the laboratory.

The duration of this experiment will typically be no longer than 14days.

All protocols have been refined so that the minimum experimental duration and animal numbers are used to achieve the scientific objectives.

What are the expected impacts and/or adverse effects for the animals during your project?

Rodent models of thrombosis are commonly used for the study of this condition and the surgical procedures and induction of the thrombus are well tolerated by these animals, as collateral vessels allow blood flow to bypass the obstruction to blood flow caused by the thrombus. The protocol for this procedure has been classified as 'moderate' as a surgical procedure is involved, but the well-being of the animals is unaffected as they are seen moving around the cage, grooming and feeding and drinking normally shortly after the operation. Pain following surgery will be minimised by treatment with an appropriate analgesic.

The use of mice in which specific genes have been removed or manipulated to be switched on or off, and agents that induce conditions that are associated with increased artery narrowing leading to thrombosis (e.g. fatty deposits in the artery wall-atherosclerosis) are also widely used in investigations of thrombosis. The genetic manipulations and agents used to promote the build-up of fatty deposits in arteries may result in a small number of mice showing adverse effects such as skin irritation that can lead to skin ulcers. Those animals that do not respond within a few days of treatment will be humanely killed.

Bone marrow transplantation between mice with different genes enhances our understanding of the importance of cells from the bone marrow (e.g. inflammatory cells) carrying those genes on processes involved in thrombus formation or its resolution. While

mice tolerate ablation of their bone marrow by radiation reasonably well, it exposes them to the possibility of infection (immunocompromise). For this reason, irradiated mice are housed in independent ventilated cages during the time they are immunocompromised (up to 21days). Prophylactic measures (e.g. treatment with antibiotics) will also be undertaken to prevent the risk of infection. The mice tend to lose some weight after irradiation, but most recover the lost weight within a few days.

Animals used as cell donors (from the abdominal source) may show adverse effects such as the build-up of fluid in the abdomen leading to abdominal tenderness on handling. Mice displaying this condition will be immediately humanely killed, and the cells that have accumulated in the abdomen removed for our investigations.

In animals having surgery (e.g. for thrombus induction or implantation of a mini-pump), incision wounds may rarely become infected or the stitches breakdown (expected in <1% of animals). These can be treated with antibiotics or resewing of the wound. If these treatments are not effective, the animals will be humanely killed.

All animals will be humanely killed at the end of an experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities and proportion of animals from all experimental protocols experiencing each severity includes:

Mild – ~ 62%

Moderate - ~38% (surgery carried out)

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 processes of how thrombosis forms in blood vessels and is removed naturally by the body, involve dynamic interactions between a myriad of molecules and cells that are impossible to replicate in the laboratory (in vitro). It is not ethical to study the effect of injecting substances and cells into patients with disease of their blood vessels without preliminary evidence that this form of intervention may be of benefit. We must therefore to carry out our initial experiments in animal models of thrombosis to allow us to study the cellular and molecular responses and interactions that take place during thrombosis and its natural resolution. We also use these models to assess the effectiveness of treatments

targeting specific mechanisms highlighted by these studies (and studies in patients with clots), on the formation and natural removal of these clots.

I am satisfied that the potential gain, in terms of treatment of human disease, is sufficient to justify the use of animal models.

Which non-animal alternatives did you consider for use in this project?

We will also use laboratory-based methods such as a flow chamber model of thrombosis that we have optimised in our laboratory to screen and assess the activity of cells and agents that we believe from our human studies (running alongside our studies in rodents) may be important in inhibiting clot formation and stimulating its resolution.

Why were they not suitable?

Studies in the laboratory cannot truly replicate the complex, dynamic interactions that take place between the many factors and cells that are involved in the formation of a clot or its natural removal by the body.

We will also develop 'test tube'- based analytical methods for the laboratory screening of cells or agents highlighted by our human studies as potential regulators of thrombosis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals required for our experimental studies based on our previous experience of the variability in the end-point measures (e.g. thrombus size, composition) in the thrombosis models, and of breeding requirements for use in these models.

The proportion of animals we expect to use over the 5yrs of this project will be:

~60% - used in breeding and maintenance

~36% - used for the thrombosis models

~4% - used as cell donors

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our extensive experience with these models in a variety of wild-type and genetically modified strains of mice has provided us with a good analysis of the variability in the endpoint measures that we have developed with these models, and we have used this

experience to carry out appropriate statistical power calculations for the numbers of animals required in our experiments.

Where we find ourselves using a strain of animal or treatment that we are unfamiliar with, or there is little data in the literature on, then we will carry out pilot experiments using longitudinal or dose escalation analysis in small cohorts of animals in order to provide statistical data that allows animal number estimations for definitive larger experiments.

In experiments that intend to understand the effect of thrombosis on e.g. vein valve function, we will induce thrombosis in a valve located in one of major veins that return blood from the limbs back to the heart (femoral and external iliac vein). This provides us with the possibility of assessing the effect of thrombosis on valve function compared with a normal valve on the vein returning blood to the heart from the opposite limb, essentially acting as an internal control. Where possible we will also use imaging techniques (such as ultrasonography or MRI that allow measurement of thrombus in the same animal over time (longitudinal imaging), thereby reducing the numbers of animals required for experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will always carry out pilot studies using small cohorts of animals prior to embarking upon a new experimental plan. We also work with other scientists who use the thrombosis models and are international experts in their field. We will discuss our experimental strategy with them to optimise the number of animals we use and determine whether tissues and data can be shared between our groups.

We will also use colony and breeding management software to help achieve efficient breeding of our genetically altered animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Species: Mice and rats are the lowest mammalian group in which models of thrombosis have been successfully characterised and are therefore the species most often used in the study of this condition. In our experience rodents tolerate the formation of thrombus in their blood vessels well, as collateral vessels spontaneously develop to bypass the thrombotic obstruction. Mouse models have the advantage that they also allow us to test the effect of specific cells of mouse or human origin in thrombosis without rejection; as well as facilitating investigation of the importance of specific genes, through genetic modification in this species.

Animal models: Rodents tolerate surgery well, with good recovery rate and low post-operative infection. Existing smaller blood vessels (collaterals) increase in their size and

quickly establish blood flow around the thrombotic obstruction. Animals are seen moving around the cage and feeding, drinking and grooming normally within 24hrs of the operation and for the remainder of the experiment's time course.

The imaging techniques to be used to evaluate thrombosis (e.g. ultrasonography, computerised tomography (CT) and MRI) have been well characterised in our institution and will be carried out under general anaesthesia. The biochemical or histological techniques used to assess thrombus composition are widely accepted for use in rodent models and will also be carried out on blood and tissues removed from the animal under general anaesthesia that terminates the life of the animal at the end of the experiment. Thrombus formation and its natural resolution needs to be assessed between 2-3days after induction (when thrombus formation is at its maximum) and 4wks (during which the process of its natural removal takes place in rodents). These time restrictions preclude assessment under terminal anaesthesia.

Why can't you use animals that are less sentient?

Less sentient animals cannot be used as they do not have a vasculature that replicates that of man or allow interrogation of the complex processes giving rise to clot development or its natural resolution. Mouse models also have the advantage that they allow testing of the activity of cells of human origin without rejection as well as allowing assessment of specific genes and disease interactions through genetic manipulation in this species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pain relief is given during and after the operation, to minimise suffering, in much the same way that we treat patients. Animals will be monitored daily at early stages post-surgical interventions and at least 2- times weekly thereafter.

Inhalation anaesthesia will be used wherever possible to minimise transient pain and distress, e.g. during surgery and imaging. In addition, full recovery between periods of anaesthesia, rehydration during long imaging sessions, respiration/cardiac function monitoring, body temperature monitoring/maintenance will be conducive to animal wellbeing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow established published guidelines to ensure experiments are conducted in the most refined way. These includes:

The Responsibility in the Use of Animals in Bioscience Research guidelines produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

Accepted limits of volumes and frequencies when administrating compounds and anaesthesia under guidance of the administration of substances. (https://journals.sagepub.com/doi/abs/10.1258/0023677011911345).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed by updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) website and seminars on the 3Rs organised within and outside of our institution.

We will also have direct support and contact with an NC3R's regional Programme Manager who supports the application of the 3Rs in our and other major institutions and is a member of the Policy and Outreach Group. This includes providing expert advice and coordinating the sharing of best practice.