Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted January – June 2021

Contents

1. Defining the role of MYBL2 in Breast Cancer Progression

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Breast cancer, stem cells, patient derived xenografts, metastasis

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Our aims are (i) to define how MYBL2 levels contributes to stem cell function in vitro and in vivo; (ii) to decipher the mechanism/s by which MYBL2 regulates survival, drug resistance and movement of breast cancer stem cells (BCSCs) between tissue layers, and (iii) to exploit this knowledge to determine if manipulation of MYBL2, and/or downstream pathways, is a way to block BCSC function and hence cancer progression.

To achieve these aims we will:

1. Defining the effect of MYBL2 downregulation in BCSC function in vivo.

2. Evaluate whether altering MYBL2 levels and/or downstream pathways could be a way to eradicate the BCSC compartment thereby reducing metastatic dissemination and disease recurrence.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 women in the UK. Despite the decline in mortality thanks to both therapy improvements and earlier detection, 90% of deaths in breast cancer patients are a result of cancer metastasis and drug resistance It has become increasingly clear that within the whole tumour, a small population known as breast cancer stem cells (BCSCs), are responsible for tumour initiation, therapy resistance and metastasis. The current clinical management for non-responders is at most palliative, aiming to prolong life and reduce pain in patients, thus, there is a real need for a better mechanistic understanding to facilitate the design of new therapeutic strategies that not only treat, but prevent metastatic, drug resistant disease.

MYBL2 is a transcription factor amplified in breast cancer, with the highest MYBL2 overexpression observed in more aggressive breast cancer types (triple negative basal breast cancer). MYBL2 amplification is associated with worse overall survival, and worse disease-free survival.

Functional validation of MYBL2 within BCSC, and the identification of drug combinations, could lead to the development of novel therapeutic strategies that specifically target this population, helping to prevent disease progression and significantly impacting on the lives of cancer patients.

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

New information on how cancer originates

New information on mechanisms that govern drug resistance and metastasis

New insight on breast cancer which potentially could facilitate the generation of novel targets for therapeutic intervention.

Potential new treatment strategies (through industry engagement)

The new information obtained through this project will be disseminated via publications and presented at cancer symposiums.

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

MYBL2 is a protein highly expressed in breast cancer and associated with short overall patient survival and short disease-free survival, and this work will be the first to investigate MYBL2 within the breast stem cell/progenitor population.

By taking a multidisciplinary approach combining in vivo mouse models with the analysis of human tumours and patient derived xenografts (PDXs), data generated will provide significant new knowledge for academics working on DNA-repair, cell-fate plasticity and cancer stem cell biology disciplines.

Long-term: Functional validation of MYBL2 within BCSC, and the identification of drug combinations, could lead to the development of novel therapeutic strategies that specifically target this population (through engagement with industry), helping to prevent disease progression and significantly impacting on the lives of cancer patients.

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

The experimental data generated by this proposal, the final conclusions and supporting graphs/tables/illustrations are useful to the wider research community, which will be shared by timely publication.

Often negative data is useful to prevent others unknowingly repeating unsuccessful experiments. Raw data is also helpful to those wishing to repeat work or use similar approaches as the info can be used for power calculations.

In addition, data will be disseminated by seminar and presentation at national/international conference. Data will be made publicly available within 3 months of a manuscript that uses the data being published.

Species and numbers of animals expected to be used

- Mice: Immunocompromised mice (1000 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.

We use mice to enable our understanding on how cancer develops and thus identify novel targets for therapeutic intervention. Virtually all the progress that has been made in understanding and treating cancer, including mechanistic understanding of cancer development and how to treat it has been done using mouse models. Mice are the chosen species because they are easy to genetically manipulate, are small and produce large litters, and easy to handle during experimental procedures. Importantly, mice are remarkably similar in genetic make-up to humans, thus mouse models of cancer will help us understand the human disease.

Moreover, the specific strain of mice that will be used under this license (immunocompromised mice) are able to grow human tumours as xenografts without human cell rejection, enabling drug testing and in vitro genetic manipulation before implantation. This enables us to mechanistically interrogate tumour growth, and hence identify new way in which to drug target. We choose to use adult mice of similar age range to minimise variability.

Another reason for using mice in our studies is that the majority of cancer models we will be using have been extensively characterised previously and develop tumours with similar aetiology and molecular profile as that observed in human disease. Published data from others inform and help to design our studies meaning approaches are more refined and use less animals. Tumour growth rate is predictable, thus less mice are required to be used for each experiment.

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

We will inoculate human cancer cell lines or patient derived tumour material subcutaneously into immune compromised mice to allow tumour growth. If we are using oestrogen receptor expressing breast cancer cells, then mice will require a minor surgical procedure to implant a slow-release hormone pellet/mini-pump enabling cancer cell growth. In some cases, when studying breast cancer stem cells (BCSCs), we will need to conduct IVIS imaging to analyse the fate of BCSCs, involving IP injections of luciferin and general anaesthesia for imaging.

Some of the cells injected will be manipulated in vitro. To be able to switch on inducible genetic elements in cells that have been engrafted into mice, we will need to feed animals doxycycline in their food.

An important part of this project is the assessment of DNA damage chemotherapies and small molecule inhibitors to target proteins related to invasiveness. The specific protein that we would like to study (MYBL2) has been linked to both DNA damage and invasiveness properties. Here, the route of drug administration depends on the specific agent but could include multiple rounds of intravenous or subcutaneous injection, or by oral gavage, or through the addition to the diet or through drinking water. To check that chemotherapies are not adversely affecting mice, we will take blood samples to test for anaemia and leukopenia. In some cases, the course of treatment will be interrupted to see if tumours grow back. Additionally, these cells could be also taken and injected into mice to study their metastatic/invasive potential.

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

Immunocompromised mice will be engrafted with human cells that will form tumours as a xenograft, the main adverse effects will be related to this.

Generation of tumours under the skin (subcutaneous model): Implantation of cells will be performed under general anaesthesia. Mice could suffer mild discomfort if not at sufficient levels, overdose- induced non-recovery, or post-operative infection at site of implantation (<1%). During tumour growth, it is possible that some mice will develop tumours that will lead to signs of distress. Although relatively

rare, this may include a 20% weight loss, failure to respond to gentle stimulation, lethargic, abdominal distension, jaundice, piloerection, intermittent hunched posture, diarrhoea, or continuous laboured respiration. At first signs of any of these the mouse will be humanely killed.

Generation of tumours in the lung (Metastatic model): Tail vein injected animals could suffer from breathing difficulties. Typically, this could occur between 5-8 weeks after injection depending on the number of cells used and the type of cell line. Animals will be culled up to 12 weeks after transplantation or immediately if they display humane endpoints such as >15-20% weight loss, body condition score <3/5, failure to drink or eat over 24 hours etc.

It is possible that delivery of chemotherapeutic drugs will cause adverse effects. As the drugs we will use are well reported in the literature, we will be able to pay close attention to the development of any signs of distress. For example, anaemia and leukopenia are common side effects of chemotherapy, and we will be able to monitor the extent of this by blood analysis.

Other small drugs do not seem to have any toxicity as reported by previous groups.

Expected severity categories and the 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 100 % of the mice will develop tumours for which the maximum severity is moderate. However, 50% of these animals will receive potential therapies which, depending on success, would reduce the 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 vitro cell culture-based models fail to recapitulate the complex interplay between cancer cells and the tumour microenvironment. New emerging approaches such as 3D cultures have emerged, but still, they are in vitro approximations that cannot reproduce the complexity of a disease. Studies using mouse models have been and still are fundamental and enlighten our understanding of the processes that lead to cancer progression and metastasis that could not be achieved otherwise. Thanks to the use of animal models of cancer, new drug targets and insights into the molecular mechanism of disease are coming to light.

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

All our in vivo work will be preceded by extensive in vitro work. This system includes culturing human breast cancer cells in monolayer as a homogeneous population on plastic, and in 3D cultures (mammospheres). However, to validate our in vitro experiments, it will be necessary to extrapolate our findings to an in vivo setting, where microenvironment plays a big part on cancer cell behaviour.

Why were they not suitable?

As explained above, in vitro systems (both, normal cultures, and 3D cultures) can give a good approximation about the activity of specific drugs on cancer cells. Nonetheless, the

in vitro cultures are far from representing the true environment of the cancer cells. The cross-talk between environmental factors and cancer cells is lost in these in vitro cultures, representing a big limitation if results are to be used for therapy. Thus, modelling cancer in mice is necessary to fully understand disease progression and identify novel therapeutic approaches.

Reduction

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

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

Animal numbers will be determined by pilot studies and estimations based on previous data either from the group, or published data.

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

NC3R's Experimental Design Assistant online tool and a sample size calculator online tool, as well as our local biostatistician will help with experimental design to reduce animal numbers, methods to reduce subjective bias, and appropriate statistical analysis without compromising the scientific objectives. Non-invasive in vivo imaging and quantification techniques of transplantations will enable multiple measurements on the same animal over a period (longitudinal sampling), reducing the number of animals, maximizing the information obtained per animal, reducing variation, and improving quality of data produced.

Where possible, transplantation experiments will be performed by the injecting two contralateral flanks of the mouse, thereby reducing the number of animals being used by a half. This will be done in particular when expanding tumour tissue for further use, or if putting experimental (+ shMYBL2) and control (-shMYBL2) into the same animal if both tumour cell types are to be exposed to the drug.

To calculate frequencies of stem cells in cancer cell populations, the L-Calc program is used. The minimum numbers required to obtain estimates of stem cell numbers with this program are data from three different cell dilutions, each of which was transplanted into five different animals. However, five dilutions into five animals are required for robust data with smaller 95% confidence intervals.

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 animals will be done responsibly and with careful colony management, ensuring breeding produces cohorts of mice suitable for experimental use.

Pilot experiments with small number of animals (n=3) will be performed in those cases where effect size cannot be estimated based on our in vitro data, the literature, or previous data from co-applicants or collaborators.

All tissue surplus to requirement will be deposited into SEARCHBreast (https://searchbreast.org), a resource to facilitate sharing of archived material derived from in vivo breast cancer models.

Refinement

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

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

Immunocompromised mice will be used, as they will allow for the engraftment of human cells without human cells being rejected by the immune system of the mouse. We will monitor tumour growth during time through the use of non-invasive techniques, reducing the number of animals required for the experiment. The tumour volume as determined by non-invasive calliper measurements or bioluminescence imaging will be plotted against time. This design offers the advantage of determining significant differences between tumorigenic growth potential of cell lines before the limited tumour volume is reached.

Why can’t you use animals that are less sentient?
The nature of the project requires that mice are in good health and alive so tumour cells can engraft and tumour can develop, thus mimicking what happens in humans. Because tumours require time to engraft, we cannot use immature Life stages or terminally anaesthetised animals/

Compared to other less sentient species such as Zebra fish or Drosophila melanogaster, which show a 60% homology with the human genome, mice and humans shared 97.5% homology of their coding DNA sequences. Like humans, mice naturally develop diseases, including cancer.

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

When conducting a surgical procedure, we ensure that analgesia is administered before the start of the procedure. Mice are allowed to recovery from the surgery by being housed in a warm cage and observed until animal has fully recovered and is mobile. Mice are checked again for wellbeing and wound closure 4hrs later. Based on experience, it has been found that by placing the wound towards the bottom third of the spine with a combination of suturing and glue, mice are less likely to bite the wound and cause reopening. Thus, we will follow this procedure on our work.
Once mice start to develop tumours, they are monitored at least twice a week by non-invasive methods, and more so if tumour growth develops rapidly.

When administrating drugs, we will use refined techniques where possible: use of flexible gavage needles, giving doxycycline in diet rather than via injectable route etc

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 P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA; Committee of the National Cancer Research Institute. Br J Cancer. 2010 May 25;102(11):1555-77. doi: 10.1038/sj.bjc.6605642.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.)

Jones HRP, Oates J, Trussel l BA (1999) An applied approach to assessment of severity. In: Humane End points in Animal Experiments for Biomedical Research (Hendriksen CFM, Morton DB, eds).

London: Royal Society of Medicine Press, pp 40±7.
In publications, we will report our animal research according to the ARRIVE guidelines and conduct our experiments with advice from the PREPARE publication (PREPARE: guidelines for planning animal research and testing. Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017 Aug 3.PMID: 28771074).

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

We will comply with the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments; www.nc3rs.org.uk/arrive), a NC3Rs-developed checklist of the essential information that should be included in publications reporting animal research. ARRIVE has now been endorsed by more than 400 journals including the Nature group, PLoS, and Cell, as well as funders, universities, and learned societies.
I will sign up for the NC3Rs newsletter, so will be made aware of any notification.
I will attend seminars organized by our animal facility aimed to keep PPL holders informed on new advances in the 3Rs.

Any new advancements will be made clear to members of my team through our weekly lab meetings.

2. Comparative Cardiovascular Homeostasis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

blood pressure control mechanisms, salt sensitivity, kidney function, podocyte function, kidney response to disease

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to further our basic understanding of blood pressure control and the development of hypertension through our studies on genes related to blood pressure maintenance. We wish to explore three key areas of kidney function - changing function of cells in response to blood pressure increases, injury-response of specialized filtration cells called podocytes and diseaseresponse of specialized cells of the kidney tubule.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 development and underlying causes of hypertension are not fully understood. High blood pressure is affected by genes and by factors like dietary salt and calorie intake, both of which are high in most developed countries. Often there is little clue to the underlying cause(s) of the high blood pressure – why are some people more affected by salt than others; how does diet during pregnancy affect blood pressure in newborn babies and adults; what key changes at the gene or cell level lead to gross changes of blood pressure at the whole body level?

This project covers rodent and fish models designed to increase our basic understanding of the complex interactions between genetic and environmental factors, which contribute to normal blood pressure control, and how imbalance leads to hypertension. The kidney is a critical organ for salt handling and will be a key focus of this project. Novel data and insights will be obtained regarding blood pressure regulation, salt balance, and the mechanisms underlying kidney damage and repair.

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

We will make our results available to the wider scientific community, through publication in peerreviewed journals and at conferences.

We will generate animal models and in vitro models, which will be made available to the wider scientific community.

We expect to identify mechanisms underlying reversible changes in cell function, and podocyte injury, which will be important moving forward to develop organoids in vitro.

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

The scientific community will gain immediate and long-term benefit from new mechanistic insights through publications in peer-reviewed journals, dissemination at International

meetings, direct collaboration and sharing of models generated. Animal models, generated through our previous work, continue to be used worldwide for essential research.

Mechanistic insights will ultimately inform clinical practice beyond the life of this project. We work closely with medical colleagues and where necessary, have access to patient materials in order to assess application to the bedside (e.g. current studies of reversible renal cell function). Pharma is increasingly engaging with universities as strategic research partners, with reciprocal benefit. We encourage links with industrial colleagues, in order to assess the action of experimental drugs in our animal and in vitro models.

Dissemination of the research, through public engagement, drives greater public understanding both of our group/projects and of the wider health issues.

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

We will maximize the output of any new knowledge, through publications in peer-reviewed journals, dissemination at International meetings, direct collaboration and sharing of models generated.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 6000 (5000 larvae, 1000 adults)
- 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.

This project will use mouse and fish models designed to increase our basic understanding of the complex interactions between genetic and environmental factors, which contribute to normal blood pressure control, and how imbalance leads to hypertension and/or kidney damage and repair.

Rodent models allow us to investigate the development of salt-sensitive hypertension by addressing the effects of dietary manipulation, and identifying key changes at gene, cell, tissue, organ and whole body level, throughout development. This requires tissue sampling at embryo, neonate, juvenile and adult stages, and dietary manipulation post weaning.

Zebrafish share gene, cell, tissue, organ and system functions with higher vertebrates. Because larvae are transparent, we are able to study early pronephric development before free-feeding is established. From two weeks of age, the mesonephric kidney develops, giving us access to additional comparative materials. Zebrafish also have the ability to repair injury, allowing us to investigate injury and repair mechanisms, both in unprotected and free-feeding stages of development.

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

Initially, we will validate our novel mouse model, by feeding a high salt diet to mice with surgically implanted telemetry devices, which record real-time blood pressure in conscious, freely moving animals. We will then collect tissue samples at key time points during which salt-sensitive hypertension develops, for further analyses, including primary cell culture and single cell RNA sequence analysis, which will provide us with large amounts of information regarding the sequelae of salt-induced changes in our cells of interest.

We have used state-of-the-art genetic techniques to manipulate gene function in a key gene involved in blood pressure control in zebrafish. Gene knockout, in a zebrafish line expressing appropriate cell reporters, allows us to analyze the consequences of gene knockout, through tissue analysis, cell culture and single cell RNA sequence analysis, which will provide us with large amounts of information regarding the sequelae of loss-of-function in our cells of interest, without the need for drug treatment. Only a small number of fish will be treated with drugs to compare the effect of drug response to loss of gene function.

We have generated zebrafish lines expressing fluorescent reporters in podocytes - key cells in the kidney, which are adversely affected in kidney injury. We induce injury or cell ablation in the podocytes of unprotected larvae, using drug treatments or optogenetic approaches, and follow the immediate response to injury/ablation in unprotected larvae.

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

Most GA rodents covered by this PPL will be bred for tissue isolation, FAC sorting, primary cell culture and/or scRNAseq analyses (sub-threshold).

Mice that undergo surgery (under deep anaesthesia), may experience pain post-operatively, but this is managed by the administration of pain-killers. Mice generally recover quickly from surgery, regaining any weight-loss within 5 days. Any weight loss observed in metabolic studies usually resolves within 4 days.

Drug treatments e.g. BrdU will be short term exposure only.

Most GA zebrafish covered by this PPL will be bred for tissue isolation, FAC sorting, primary cell culture and/or scRNAseq analyses (sub-threshold).

Zebrafish that undergo drug treatment (e.g. captopril for tissue challenge) or injection of drugs (e.g. puromycin for cell ablation) may suffer from oedema to a level that will not affect survival.

Expected severity categories and the 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 undergoing surgery, dietary manipulation or drug challenge are likely to experience moderate severity of experience. Only 10% of all animals are likely to experience this level of severity.

The vast majority of zebrafish that undergo injection of drugs for tissue injury or cell ablation will be less than 5 days old (unprotected) and are unlikely to suffer because of immature sensory perception.

Captopril will be administered to adult fish at a dose level that does not cause adverse effects.

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?

Hypertension is a highly complex disorder that cannot be replicated in a culture dish. It involves multiple organs, hormones, genetic, neuronal and environmental factors. By undertaking basic research into specific aspects of gene function, metaplastic differentiation, cell plasticity, injury and repair, we can understand the complexity of the kidney structure and function. By developing in vitro models of these processes, we aim to work towards the development of functioning kidney organoids.

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

We have considered primary cell isolation, characterisation and culture in all aspects of our current research. Inconsistencies between presently available cell lines, such as the $mCCD_{cl1}$ cell line, and their equivalent cells in vivo, mean that observations may have limited application in the clinical setting. For example, mCCD cells do not appear to replicate the full degree of plasticity between PC, ICalpha and ICbeta cells. This project will enable the study of more appropriate cells ex-vivo.

Why were they not suitable?

Presently available immortalized cell lines may not be as suitable or informative as primary cells. We are therefore planning to isolate primary cells, wherever possible, to use for in vitro analyses.

Reduction

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

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

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

We estimate the minimum number of animals required to achieve 80% statistical power in each experiment. This is judged using data from closely related experiments in the Centre or in recent scientific literature. We routinely monitor variance during experiments allowing us to adjust numbers pro-actively and use the minimum required for robust statistical outcome.

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

Where possible, factorial designs are used to maximize the information obtained from the experiment. For example, next generation sequencing analyses maximize the information attained from a small number of animals.

Longitudinal rather than cross-sectional studies considerably reduce the number of animals needed for time-point evaluation in, e.g. disease progression and remission; in vivo studies (metabolic cages) and telemetry recording of blood pressure. Repeated in vivo imaging reduces the need for time course experiments in which multiple animals are culled at different time points and the tissues examined histologically. These procedures provide a statistically powerful (repeated measures) approach to integrative phenotyping and importantly direct other studies to informative time points.

Collaboration with experts in relevant specific techniques, ensures success and reduces animal numbers. We will import relevant rodent and zebrafish lines from collaborators or commercial breeders where appropriate.

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 maintain the minimum numbers of rodents and fish in each line to allow propagation in order to supply sufficient animals for 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.

Rodent transgenic strains are required to model clinical conditions, and are critical for comparative purposes regarding cardiovascular, metabolic and renal function. Since genetic manipulation is advanced for these species, we constantly refine strategies for gene targeting, such as CRISPR technology, (using targets identified by microarray analyses) to maximise information obtained from transgenic models.

By inducing gene expression through, for example, time-limited exposure to pro-drugs, any adverse effects are minimised. Surgeries will be combined where possible. Animals undergoing any surgical procedures are monitored closely by a technician/researcher familiar with the model. Analgesia is administered post surgery as advised by the NVS or NACWO.

Tailored monitoring, and the implementation of strict humane endpoints represent significant refinements.

We increasingly use zebrafish larvae to understand the role of genes such as podocin in early kidney development. Most observations are made before larvae are free-feeding. The short generation time and optical clarity make the zebrafish an attractive organism for these studies, and this will significantly reduce the number of rodents, which would otherwise be used under this PPL.

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

Where possible, we routinely use the less sentient zebrafish models.

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

We will strive to use the minimum number of animals in protocols of moderate severity, while achieving statistically significant results. We constantly strive to improve surgical and post-operative care and monitoring schemes. We will routinely introduce nesting materials and stimulae to home cages.

Similarly, for zebrafish, we strive to reduce startle responses and stress while netting the animals.

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

We will observe best practice guidance as specified by the NC3Rs and the latest FELASA guidelines for rodent and zebrafish use.

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

We will, throughout the project, follow the latest guidance given by our local NVS, the NC3R newsletter, discussions with colleagues and literature review. We will also follow the latest FELASA guidelines on rodent and zebrafish use.

3. Cortical and subcortical networks for rodent cognition

Project duration

5 years 0 months

Project purpose

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

Key words

cognition, cortex, memory, rodent, thalamus

Animal types	Life stages
Mice	juvenile, adult
Rats	juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim is to identify how specific interactions within the rodent brain support different aspects of cognition. A particular focus concerns two brain regions (the anterior thalamus and hippocampus), which are connected to each other and are both associated with neurological conditions causing memory problems.

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

Why is it important to undertake this work?

Numerous common neurological conditions permanently impair cognition, including

memory. Notable examples include strokes and dementia. The conventional focus for the study of memory has been the status of medial temporal brain sites (the hippocampal formation and parahippocampal region). Our programme of research has repeatedly highlighted how this focus is detrimental as it ignores a network of cortical and subcortical sites upon which the hippocampus depends, while also showing how these same sites have additional cognitive functions. The planned level of analysis (at the neuronal level) and the lack of selective neuropsychological data for some of the key sites (e.g., the anterior thalamic nuclei) has meant that animal-based research provides unique insights. While neuroimaging findings from humans complement the animal data, they lack anatomical resolution, resulting in an absence of findings at the neuronal level.

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

The principal scientific benefits come from new information leading to a clearer understanding of cognitive mechanisms. The principal outputs will be communicated in scientific publications and presentations (including those to non-specialist audiences). Over the past five years we have published over 40 papers from research under by my last PPL, reflecting our effectiveness at disseminating new information. Additional outputs include open access to our experimental data from the time of publication.

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

In the short-term, our studies will generate a better understanding of distributed neural systems for learning, memory, and attention. The main beneficiaries will be academic researchers in closely related fields. This outcome will principally be delivered by the papers published across the timescale of the PPL. Evidence of the current impact of my research can be seen in my overall scientific citations (>10,000 since 2015, i.e., from the date of the start of my previous Project Licence).

Over the longer term I will continue my collaboration with human imagers to examine some of the basic tenets of the models we are developing and to maintain the two-way flow of information from animals to humans and back again. This approach reflects my determination to ensure that our discoveries in animals can help recast and refine questions about human memory and the causes of memory loss in conditions such as dementia. Past examples include the discovery that recall and recognition can operate using different brain circuits, with implications for both animal models of dementia and amnesia, and for the testing of initial signs of dementia, e.g., in Mild Cognitive Impairment.

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

I will continue my collaborative approach to neuroscience, both within the UK and beyond. Other collaborations involve human imaging, where findings from my animal research help guide the collaboration.

I have a strong track-record of academic publications (250+ papers, >30,000 citations) and conference presentations. Our group will continue to publish papers in open-access form to enable the widest readership.

We will, also use forums such as BioRxiv to make pre-prints accessible, so alerting other researchers to our findings and, thereby, stopping duplication, while also providing access to our research data.

I have confidence in the value of published null results (subject to appropriate methodology and power). One valuable vehicle is the new, open-access journal Brain and Neuroscience Advances (launched 2016). One feature of this journal is its willingness to publish well-executed research with null results and/or failures to replicate.

I am registered to ORCID to increase the availability of my research output.

Species and numbers of animals expected to be used

- Mice: 250
- Rats: 1450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 preferred choice. They are used because they are mammalian species that afford translation to humans, allow the implementation of targeted genetic manipulations, and display high levels of behavioural flexibility needed for the experimental procedures to be implemented. A further criterion is that the key brain sites retain the same basic structure and connectivity between rodents and primates.

Further benefits of studying rats and mice stem from the wealth of past neuroscientific findings concerning these species, ensuring that there is no need for additional studies to test basic aspects of structure and function.

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

A total of 1700 animals (1450 rats, 250 mice) will be used across the life of the project. The purpose is to understand the properties of a set of interconnected brain regions that form a network in the rodent brain to support cognition. The principal objective has three components.

To understand the anatomical properties of the network, adult mice or rats (approximately 10% of animals) will typically receive a general anaesthetic (gas) to initiate a surgical procedure. After creating a small hole in the skull, it is possible to inject very small volumes of one or more tracers into target brain sites. Following wound repair and recovery the animal is cared for until the tracer has moved along neurons (days or weeks depending on the tracer).

To map the location of the tracer the animal is anaesthetised and then a fixing solution is injected into the blood stream via the heart (‘perfusion’), and the brain removed after death. To understand the functional properties of the network, some studies (approximately 75% of animals) will manipulate a specific target site or its connections and determine how that affects cognition, as measured by behavioural analysis. Adult mice or rats initially receive a general anaesthetic (gas) as part of a surgical procedure (or pair of procedures) that involve making small holes in the skull, allowing the injection of viruses that travel within neurons, without harmful effects. Following recovery, injections of a drug to activate the virus makes it possible to temporarily moderate specific pathways

('chemogenetics'). These drugs might be injected systemically (into the body) or intracerebrally (into target sites in the brain). Alternatively, we will inject a viral construct that is light sensitive, so that it can be switched on and off by local light sources placed on or within the brain, thereby, switching neurons on and off ('optogenetics'). The effects on cognition are measured by behavioural tests of learning.

Some of these behavioural tasks assess spontaneous patterns of activity, e.g., object recognition, while others may involve the use of food (rats) or liquid (mice) rewards. For these studies, food or water (but never both) may be restricted under carefully monitored regimens with well-defined criteria. On completion, the brain is studied following perfusion and removal. Such studies typically last for 3-6 months.

To understand the neuronal signalling properties of the network, studies will record the electrical activity of multiple sites. Adult mice or rats (approximately 15% of animals) initially receive a general anaesthetic (gas) as part of a surgical procedure that involves making small holes in the skull, allowing microelectrodes to be fixed and retained in target sites. Following recovery from surgery, neuronal recordings are made in awake behaving rodents performing tasks known to tax specific aspects of cognition. In a further refinement, these recordings may be combined with either 'chemogenetic' or 'optogenetic' manipulations (see above), in order to detect how pairs of sites interact. On completion, the brain is studied following perfusion. Such studies typically last for 3-6 months.

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

For studies understanding the anatomical properties of the network, full recovery from the surgery is expected and the injected tracers themselves have no painful actions or effects on behaviour.

Consequently, the transient adverse effects (pain) arise from the immediate effects of the surgery (skin incisions and sutures).

For studies designed to understand the functional and neural signalling properties of the network, the adverse effects of any surgeries are very similar to those described above, recognising that the brain itself has no pain detectors and any injected substances have no direct aversive actions. One additional factor is that some animals have cannulae or electrodes permanently fixed to their skulls so that infusions or recordings can be made over periods of weeks or months. Nevertheless, animals display little sign of discomfort and continue to show normal patterns of eating and drinking.

As the purpose of the experiments is to understand cognition it is vital that the animal can show a full repertoire of normal behaviour and have intact sensory-motor abilities.

For some behavioural tests, animals may be encouraged to seek food reward. For this reason, they may be food restricted to ~85% of free-feeding weight, a level that serves to counter obesity and has no negative health effects. In a very limited set of circumstances animals might be trained for a liquid reward, during periods of very carefully controlled and monitored water restriction. Excessive changes in behaviour or loss of body weight would, however, lead to the immediate discontinuation of experimental work and either termination of the animal or appropriate treatment under veterinary advice.

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

species.

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

Rats - 1450 overall. Expected to be 1250 moderate, 200 mild. Mice - 250 overall. Expected to be 150 moderate, 100 mild.

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

- Killed

Replacement

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

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

The research concerns the neural basis of cognition. This poses a challenge as any manipulation ultimately requires assessment or verification against some aspect of cognition, typically learning and memory, but also attention. The sheer complexity of mammalian neural networks and the need for a behavioural output measure has, so far, meant that there are no viable non-animal alternatives.

Computational modelling, while of much interest for the study of plasticity and signalling, involves networks that lack sufficient biological complexity and lack the required verifiable endpoint, cognition. While cross-referencing to the human brain is invaluable, the level of analysis that is the focus of our research simply cannot be addressed by the study of human brains.

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

Two options would be to switch to computational simulations or to conduct the research with humans through neuropsychological methods and/or using non-invasive neuroimaging.

Why were they not suitable?
Simulations: The challenge of understanding cognition in the context of real biological systems means that while computational modelling can test general principles of information transfer it cannot place these in an environment that captures the complexity of the mammalian brain. Nevertheless, we are in discussions with mathematicians to look for underlying principles governing patterns of connectivity.

But this research still demands accurate biological data.

Human studies: It is, alas, not possible to switch to human studies if we wish to understand the detailed mechanisms of thalamic – cortical interactions for cognition. Neuropsychology often relies on the analysis of patients with selective pathologies. There are, for example, no known syndromes that selectively affect the anterior thalamic nuclei. Despite the occurrence of thalamic strokes, the pattern of arteries within this brain area means that circumscribed loss of the anterior thalamic nuclei never seems to occur. (There are,

however, conditions where anterior thalamic pathology or disconnection is associated with cognitive changes – consistent with our predictions from animal research - but these conditions never affect individual brain sites.)

Neuroimaging, even at the highest resolution, struggles to identify the individual anterior thalamic nuclei and cannot isolate the functional interactions of these nuclei with fMRI. Meanwhile, diffusion MRI cannot reveal the direction of any given nerve pathway (i.e., distinguish outputs from inputs) nor can it reveal diffuse pathways. Furthermore, neuroimaging studies are very often correlational in nature, making it very hard to identify causality.

Despite, the many limitations of in vivo imaging of the human brain, I will continue to collaborate with experts in these techniques as my expertise in neuroanatomy and cognitive networks has made it possible for me to guide and interpret collaborations using these non-invasive 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?

Behavioural studies: The research involves both conventional permanent lesions as well more-recently introduced viral-based methods (chemogenetics and optogenetics). The latter have the potential to induce transient disruptions that are more targeted (both spatially and temporally) than conventional lesions. Using these viral-based methods over recent years has resulted in reduced overall animal numbers. The reasons include the more detailed analysis of individual animals combined with the potential for a given experimental animal to be used as its own control, by switching the manipulation on and off. It is, however, still vital to have separate control groups for any nonspecific actions of the virus/activating stimulus and its surgical delivery.

Anatomical studies: We will continue to examine the fine nature of the connections to and from anterior thalamic nuclei and draw comparisons with other brain sites by tracking tracer injections. Animal numbers depend on the complexity of the sites of interest and how they might differ. The axonal transport of fluorophores tagged to viruses, e.g., for chemogenetics, does, however, mean that tissue from behavioural studies can also contribute to anatomical investigations. We are guided by the twin challenges of obtaining minimum confirmatory cases with the value of confirming any differences between brain sites. Power calculations are rarely feasible at the outset of a study. We do, however, carefully monitor the accumulation of data in order to stop as soon as is scientifically acceptable.

Electrophysiological studies: A very similar situation arises to that just described for anatomical studies. The data from individual animals can vary enormously in quantity and quality. There is a balance to be made between generating sufficient data in repeat animals in order to draw valid (reproducible) conclusions while, at the same time, not using any more than required. One consequence is the need for data analysis to proceed in

parallel with the experiments, so that an end point can be determined as it is reached.

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

Choice of species: The studies use rats and mice. One of the many benefits is that we can limit ancillary studies as there is a large literature on which we can build. For example, many of the major anatomical links have already been described and detailed using the in vivo transport of tracers injected into brain sites.

Choice of behavioural tasks: Priorities involve validity, ease of acquisition, and whether studies can be 'within' or 'between' subjects. 'Within subject' tasks (typically where the animal can act as its own control) include 'working memory' tasks in which the same problem can be given repeatedly to the same animal, with overall performance remaining stable. This approach reduces variance, so increasing statistical power and, thereby, reducing animal numbers. Over recent years we have devoted time to refining both spatial (e.g. T-maze alternation) and nonspatial (e.g., object recognition) tests of working memory.

By switching to procedures that have multiple trials in a single session for tests of object recognition and recency we can reduce data variance, so requiring fewer animals. The introduction of transient interventions, e.g., optogenetics, makes this 'working memory' approach all the more powerful, aiding the re-testing of the same animal as its own control.

Some key tests (e.g., attention-shift) cannot, however, be run as 'working memory' tasks as an individual animal cannot be re-tested on the same class of problems (as the repeat would substantially alter levels of performance). Here, the priority is for a task that can be readily acquired by all animals (to reduce variance and training duration). Digging tasks provide an excellent example as they use a natural behaviour (foraging to find buried food) where rodents are naturally biased to associate attributes of the digging material with the food. This combination promotes rapid, reliable learning.

The incorporation of complementary connectivity data: The neuronal transport of signals tagged to viruses, e.g., for chemogenetics, means that tissue from behavioural studies can also contribute to anatomical investigations. A further resource is the Allen Brain Atlas https://mouse.brain-map.org/. This on-line site contains details of hundreds of anatomical tracing experiments in mice (all involving anterograde tracers). Data from this resource complements and extends that from our rat experiments, so helping to limit the numbers of rats required.

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

Animals come from accredited sources where we are confident of their health status and homogeneity.

Trying to determine the optimal number of animals required in advance of an experiment is not straightforward. Power calculations are a valuable first step but also have limitations as they are derived from other, albeit similar, studies, i.e., effect sizes may differ between studies. Pilot studies, therefore, have an important, additional role in setting group numbers, or in determining whether any potential effect size is so small that the numbers of animals required would be ethically unacceptable. Bayesian analyses of data acquired incrementally provide a valuable check on the status of a study, i.e., whether to proceed

further.

I am an advocate of tissue sharing, e.g., for anatomical projects. We are currently collaborating with other research groups in the UK and beyond to share rat anatomical data, so avoiding duplication. As described above, we have extended this practice to use publicly available databases, where possible, e.g., Allen Mouse Brain Atlas, https://mouse.brain-map.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.

In the past we have been very reliant on studying the impact of permanent lesions (e.g. by neurotoxins) in selective brain targets and measuring their effect on behavioural tests of cognition (along with sham lesion controls). We will largely replace this traditional approach by the use of 'chemogenetic' and 'optogenetic' methods to transiently modify targeted pathways in rodents. In chemogenetics, a chemical ligand is injected which temporarily activates specific nerve sites that have previously been made sensitive to that chemical. In optogenetics, light is shone onto a specific brain area that has been modified so that light pulses alter neuronal activity. In some of these preparations there is an indwelling chemical injector/light source to target cortical or subcortical sites. Any such preparation must be fully compatible with the animal performing complex behavioural tasks in an unencumbered manner.

These newer methods are a refinement over the previous standard methodology (the making of permanent brain lesions) from both a scientific and welfare perspective. One advance is their ability to isolate specific, site-to-site interactions. The previous way to test site-to-site interactions ('crossed lesion disconnection') required a minimum of four separate surgical groups (now typically replaced by two or even one, with these new methodologies). The previous surgical groupings included animals with multiple, crossed lesions in different brain sites. That disconnection approach also had important theoretical and practical limitations, e.g., the direction of effect between the two sites could often not be inferred, while fibres that cross hemispheres can suppress any disconnection effects, causing misleading null results. Consequently, these newer methods (chemogenetic and optogenetic) require fewer groups (i.e., fewer animals) and create less ambiguous data, so limiting the need for follow-up studies.

Perhaps the largest change from a welfare perspective is the emphasis on transient, highly-localised neuronal dysfunctions. Consequently, we can ensure that for the large majority of the post-surgical period the animal has a functionally intact CNS (in contrast to conventional, permanent lesions). This transient intervention approach minimises any overt or hidden negative consequences of the 'lesion'. For the same reason, the new techniques do not cause lasting harm, e.g. to sensorimotor, emotion, or motivation states, as any impact is brief. The more targeted nature of the interventions further reduce the

likelihood of unintended consequences. These techniques also create new opportunities for within- subject statistical comparisons, which rely on comparing the same animal in two different states (brain site inactivation versus unaffected state). Statistically this is more powerful than comparing between groups, meaning that some behavioural tests can be reduced in duration. For chemogenetics the drug used to activate the injected construct has been carefully selected so that at the dose levels used it should have no nonspecific overt affects - this is critical both for wellbeing and to ensure unambiguous behavioural data. For optogenetics a 'cold', restricted light source is used.

Our goal is to understand the neural basis of cognition. Consequently, sensorimotor regions are avoided, ensuring minimal nonspecific adverse effects when the transient 'lesion' is applied. Moreover, our target sites are not thought to regulate basic levels of motivation, such as eating or drinking. Cage enrichment (chewing blocks and tubes) is typically standard, while group housing is adopted whenever possible.
Finally, the greater scientific investment in each animal has meant a shift towards greater pre-training, with extensive handling, habituation, and initial training, prior to any surgical intervention. In this way we can ensure that all animals receiving interventions can readily acquire the behavioural demands when in the control state. This practice is beneficial in reducing animal stress and aiding recovery.

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

Our research is designed to relate to the adult and aging human brain. Rats and mice remain the choice of species as the structure and connectivity of key brain sites are strikingly similar to ours. To study cognition, it is vital to examine learning tasks that are analogous to those that humans encounter. Rats are unusually versatile at problem solving, partially reflecting their omnivorous nature. They show a wide repertoire of stratagems helping us, for example, to discover and analyse the brain’s multiple spatial location and navigation systems. (The Nobel Prize in Physiology or Medicine, 2014 was awarded to O’Keefe, Moser, & Moser: "for their discoveries of cells that constitute a positioning system in the brain" – discoveries initially made in rats.) We are unaware of any demonstrably, less sentient species that has the required cognitive skill-set.

Nonrecoverable studies under terminal anaesthesia are of value as an initial first step in determining the consequences of a manipulation (e.g., chemogenetic or optogenetic) at the level of individual neurons, e.g., through electrophysiology. This will, however, only provide preliminary data as, if the full behavioural experiment is later initiated, it will be necessary to assess neural activity in awake, behaving animals. We are, however, able to use the expression of immediate-early genes in post- mortem tissue as a proxy for neural activity in some studies, so avoiding additional in vivo electrophysiological verification.

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

The Animal Health checklist (see Action Plan) provides an invaluable means to monitor and standardise the monitoring of animals. In consultation, we look for any further checks that might usefully be added to the section 'Expected Adverse Effects specific to your research, in order to reflect the novel techniques that we are increasingly using. One example concerns the status of any indwelling cannula.

A different area of anticipated refinement concerns the ligands (currently clozapine) we use to activate DREADDs (both systemically and intracranially). Recent studies suggest

alternatives with better binding properties, resulting in less spread within the brain, i.e., less potential for ‘off target’ unwanted effects that might potentially cause distress to the animal.

We also anticipate the emergence of reliable DREADDs virusest h.t travel retrogradely in nerve axons (we currently only use anterograde viruses). Retrograde transport will allow more precise targeting of subregions within sites of interest, e.g., specific thalamic nuclei. This advance should result in more selective cognitive dysfunctions, with less risk of any distress or unintended consequences to the animal.
We increasingly pre-train animals on behavioural tasks prior to surgery. This has multiple benefits. Occasional animals are unable to acquire task rules, and so it would be inappropriate for them to receive surgical interventions. Pre-training speeds up the required days of training post-surgery, so reducing time spent following intervention. It is also likely that this practice reduces stress at the time of surgery and aids recovery. We also increasingly rely on behavioural tasks that match the animals' innate predispositions, e.g., tasks based around natural foraging, while ininnatecreasingly eliminating the use of aversive stimuli.

Further routes for refinement arise from discussions with the NACWO, aided by the recent presentation of our research programme to the animal support staff. NC3Rs provides a vital source of new ideas and welfare practices, e.g., rat 'tickling', available to all our researchers.

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

NC3Rs provide a repository of information, including updates concerning Refinement. We will regularly access this information, e.g. webinars, along with receiving notifications as subscribers. The ARRIVE guidelines provide a self-checklist to ensure experimental rigour. Other relevant information is published by UFAW, FRAME, RSPCA, and LASA.

ARRIVE 2.0 (2020) has been updated and reorganised into two sets, the “ARRIVE Essential 10,” which constitutes the minimum requirement, and the “Recommended Set,” which describes the research context. Prior planning and adherence to these requirements will enable both research rigour and refinement.

Refinement also arises from awareness of current research developments (from web resources, e.g., Pub Med, Web of Knowledge, Google Scholar) and having sufficient flexibility to adapt to new, improved methods.

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

A vital method is via the NC3Rs website https://nc3rs.org.uk/ I and all my research team are registered subscribers, so we receive regular news updates and reminders of resources. Topical examples include information on the new ARRIVE guidelines (2.0, 18th August 2020). These resources are further promoted NC3Rs Regional Programme Managers.

Other relevant information comes from the Laboratory Animal Sciences Association (The Forum), UFAW (University Federation for Animal Welfare), the RSPCA, and the NVS.

Implementation arises from discussion with the research team, the NACWO and their support team, along with the NVS. To enable this process, I recently gave a seminar to the NACWO and support staff regarding the goals and nature of my research, so they are best placed to assist.

4. Investigating disorders of sex development in the mouse

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

disorders of sex development (DSD), sex determination, mouse models of human genetic disease, gonad development, cell fate determination

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?

There are two broad aims: i) to create and study mouse models of human disorders/differences of sex development (DSD); and ii) use these models and other approaches to identify molecular and cellular mechanisms by which cells in the developing fetal gonad commit to becoming a building block of either a testis or an ovary.

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

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

Why is it important to undertake this work?

Sex development (or sex determination) describes the process by which the fetus develops male gonads (testes) or female gonads (ovaries) during gestation in the womb. Usually, a chromosomally XY fetus develops testes and an XX develops ovaries. Disorders/differences of sex development

(DSD) in the human population are relatively common and can affect development of the gonads (testis and ovary), the reproductive tracts (such as the vas deferens or oviduct) and the external genitalia. Whilst sometimes DSDs can be relatively mild, as in the occurrence of undescended testes, they can sometimes, although rarely, have a more dramatic and distressing impact, as in cases of XY female presentation or ambiguous genitalia. The majority of cases of DSD that disrupt the development of the gonads (gonadal dysgenesis) still do not have an explanation at the genetic level and therefore do not benefit from a genetic diagnosis and associated clinical management.

The creation of mouse models of DSD has two major impacts: i) it allows clear roles in gonad development (sex determination) to be established for genes (and changes to those genes) implicated in human DSD by clinical and human genetics investigations - these can then be used for novel diagnostic tests for specific DSDs; ii) it reveals the genetic principles by which genes act to build mouse and human gonads, shedding light on similarities and differences and revealing mechanisms by which cells adopt a particular fate (testicular or ovarian) during fetal development. The latter component forms part of the bigger project, happening in labs all around the world, of understanding how a single-cell embryo develops into a multi-cellular organism and exploiting this knowledge in regenerative medicine (such as stem cell therapies, and so on).

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

The primary output will be knowledge: understanding the role of genes in mouse gonad development and thereby shedding light on the origins of disorders/differences of sex development (DSD) in humans, with a view to improving diagnosis. This knowledge will be the product of collaborative interactions, ongoing for a number of years, of human geneticists, clinicians and mouse geneticists, in the UK and elsewhere in the world. The knowledge will include understanding molecular mechanisms of gene function but also how genes interact. We now routinely study mice that have alterations to more than one gene and on different genetic backgrounds. The idea here is to model the more complex genetics that characterises human diseases, where evidence for digenic (involving two genes) and oligogenic (involving several genes) inheritance is becoming clearer. Therefore, one additional output here are the mouse lines that we generate (3-6), which will be of interest to other researchers nationally and internationally, both in the sex determination field and beyond. They may allow the identification of new biomarkers of disease and possible therapeutic targets.

One focus of our phenotypic work at the moment is the use of single-cell approaches. This means we can examine the impacts of deleting a gene on the expression of all other genes, one cell at a time. This approach is already revealing the complexity of the sex determining pathway and pervasive disruption to or loss of sex identity at the cellular level in genetically altered mice. The approaches we are taking will be of use to others in the field and beyond and we will advertise the computational approaches we take, and associated protocols, and make them available to others, through scientific meetings and publications.

We have a track record of publishing in high profile journals and will continue to do so. We will also disseminate novel data and methodologies through involvement at meetings and in collaborative networks in which we operate, and through publication.

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

Our immediate collaborators, especially those studying the genetics of human sex determination, will benefit from the insights that our research creates. They will be able to tailor their genetic screens and molecular analyses to reflect the knowledge we produce in the mouse. This might include investigating in humans genes related to the mouse genes we study, or genes in the same 'pathway' of activity. It will also allow them to perform functional studies on human cell lines or with human fetal tissue, using hypotheses arising from the mouse data we provide. The interaction here is synergistic and iterative: we benefit from their studies of human genetics in identifying genes to study further in the mouse, and the mouse genetics and phenotyping data allow them to further refine and extend their studies in humans, and so on.

Our mouse genetics collaborators and the wider mouse community will benefit, because we make available all of the genetically altered lines that we generate. Some of these lines will be of interest to researchers studying other systems (cardiovascular, neural etc), because many of the genes we study impact systems beyond the reproductive organs and related phenotypes may be observed.

Another group to benefit will be clinicians who are looking to close the existing diagnostic gap, such that individuals with DSD can be confidently given a diagnosis for their condition. Additionally, patients and their families may benefit from such diagnoses and the improved clinical management they permit.

Finally, the wider scientific community will benefit from the approaches that we take and novel methodologies we use, including computational methods for examining single cell gene expression in developing tissues.

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

All of our experimentation is collaborative. We only study genes that our human genetics/clinical colleagues consider interesting and worthy of further study. If initial investigations in the mouse suggest that it will not be profitable to proceed, the experiments will be terminated. We have, however, previously published data showing that a particular gene does *not* function in mouse sex determination: this is because some

genes are associated with a wealth of supporting evidence, from humans and other sources, *suggesting* a potential role, and it is important that we publish negative data (showing that such a role does not exist) to prevent others from repeating our studies. We will continue to publish such negative data.

Species and numbers of animals expected to be used

- Mice: 9850

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 aim of this project is to model aspects of human disorders of sex development (DSD) in mice and study the genetic control of gonad development. As a mammal, the mouse has reproductive organs that are similar to those of humans, in contrast to other non-mammalian vertebrates, like fish and birds, that differ significantly in the ways in which they use genes to build gonads in a fetus. More generally, the mouse model is known for its utility in reproductive biology, its contribution to reproductive medicine, and medicine more generally, as a model of human biology. Moreover, research into mouse gonad development by my laboratory and others over the last 30 years has revealed that many genes functioning in mouse sexual development also function in human sexual development. Examples of these include the key pro-testis genes SRY, SOX9 and DMRT1, and the pro-ovary genes WNT4 and RSPO1 – and this commonality strongly suggests that pathways of gene activity are highly conserved between mouse and human sex determination.

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

Typically, mice will be bred for maintenance of a genetically altered strain. Some females will be mated and killed when pregnant in order to allow the study of fetal reproductive organs. Occasionally, but rarely, pregnant mice will have a chemical agent administered to them in order to allow control of timing of gene inactivation in a fetus, activation of transgene expression or mark dividing cells.

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

Occasionally, but very rarely, adult mice may experience some lasting harm because of the novel genetic alterations that they have. Suffering here will be minimised by using refined methodologies and finding ways of targeting the impacts of the genetic alteration to the gonad: gonadal abnormalities alone are very unlikely to result in suffering.

The vast majority of the research proposed here focuses on the fetal mouse gonad, between 10.5 and 14.5 days after fertilisation. There will be very few adult mice that

experience anything other than transient discomfort, since most will only be carrying (heterozygous) mutations.

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

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

The vast majority of animals bred under this licence will experience suffering sub-threshold: they will experience no suffering other than transient discomfort. Around 10% will experience up to a mild limit and a small minority (5-10%) will suffer up to a moderate limit.

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 adult gonad is a complex organ comprising many different cell types, such as germ cells, which contribute genetic material to the next generation, and gonadal supporting cells, which support germ cells through their developmental journey, from embryonic primordial germ cells through to mature gametes just prior to fertilisation. Hormones are produced by steroidogenic cells. These cell types are already in place during the fetal period. Fetal gonad development is a complex, dynamic process, involving migration of cells from sites outside the gonad, and a complex choreography that is very time-sensitive, by which different cells types communicate and come into close proximity over time. Only the examination of this process in the context of the whole developing fetus can allow a reliable examination of the process of gonad development as it occurs during pregnancy. This process cannot yet be studied in individual cells in isolation or in groups of cells cultured together.

The study of gonadal function requires the use of a whole animal because it is subject to control by a complex hormonal system, involving the pituitary gland and hypothalamus of the brain. This system cannot yet be adequately modelled in a dish.

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

We have used cell lines to perform various functional tests and such lines will still be useful to us in future. In particular, we have used cell line assays to assess the impact of mutations and

'reprogramming' methods to turn stem cells into cells that mimic cells of the fetal gonad. But this latter method only works so far to produce one of the gonadal cell-types, and the cells are only *similar* to endogenous fetal cells *in vivo*: they do not faithfully recapitulate the features of cells isolated directly from a fetus. But they can still be useful.

In addition, we have used 'organ culture' methods to study particular processes - such as cell migration into the gonad - but this still requires an early fetus to provide an early stage gonad, which is then developed further in a dish in culture media.

Why were they not suitable?

The cell models discussed above allow certain processes, particularly biochemical processes, to be studied and we have done so and will continue to do so. But the aim here is to study the impact in the mouse of genetic alterations that model those found in humans. Studying the impact of those genetic alterations in individual cells will not allow a complete understanding of how they impact the complex, multi-cellular choreography of mouse (and human) gonad development in the fetus. The intricate ways in which cells communicate with each other over time is vital, and this cannot yet be modelled in a dish, outside of the normal environment of the developing fetus in the womb. But we continue to be vigilant about the development of 'gonad organoid' approaches i.e. 'artificial' gonad-like organs made by mixing different cell-types together. Nobody has yet made such an organoid, but some laboratories, with whom we are in contact, are trying. The development of *in vitro*-derived gametes is one reason for generating gonads in a dish, but better understanding gonad *development* is almost certainly a condition of future success.

Reduction

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

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

The numbers estimated here are based on over 25 years of studying and publishing on the process of sexual development in the mouse and the statistical methods that have underlain this. We have extensive experience in performing experiments and publishing data from them; we know how to design controls and how many repeat experiments we need to perform, in order to generate statistically significant data that will pass the peer review process prior to publication. Such publication is vital for maximising the impact of our data on the community of researchers.

As an example, 5-6 independent fetal gonads will be required to reliably examine the impact of a genetic alteration on gene expression, using a variety of techniques. The number of such experiments has been estimated based on the number of different genes whose expression we need to analyse. On average, a pregnant female mouse will provide around 8 embryos/fetuses. Only 1-2 of these might have the desired genetic alterations,

although others can act as important controls. On some occasions, the number of desired embryos may be less, such as when mice with more complex genotypes are crossed together. These sorts of consideration account for the number of adult mice that need to be bred.

Lower numbers are required for the moderate breeding licence since the vast majority of mice bred under this licence with be sub-threshold or mild.

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

We have access to, and make regular use of, a trained statistician to assist in experimental design.

We mostly use a particular strain of mouse, C57BL/6J, which is ideal for investigating impacts on male gonad development. We have enormous amounts of experience in what to expect from this strain in normal gonad development, and therefore deviations from that norm are easy for us to spot if we repeat experiments on a handful of occasions to verify the outcome. The outcome is often quite tightly constrained by using the same mouse strain, in which every individual has a near identical genetic constitution (genome). This also helps to keep numbers of animals to a minimum. Single-cell approaches have also meant that we do not have to collect large numbers of fetal gonads to purify enough RNA for bulk RNAseq approaches or protein for Western blotting.

Females intended for tamoxifen dosing will be palpated at 9.5 dpc and if not pregnant can be used for timed mating again rather than dosed and wasted.

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

We continually monitor and revise the amount of breeding we perform. Breeding is designed to ensure that we have sufficient number of female mice to support the generation of fetal gonads for use in various types of experimentation. The minimal number of phenotypic tests on fetal gonads will be performed, consistent with being able to establish convincingly that a phenotype exists and, if possible, its molecular and cellular basis. Regular meetings with the breeding team ensures breeding is matched to the number of females required for timed matings.

Refinement

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

Which animal models and methods 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 focus is on using similarities between mouse and human reproductive biology to shed light on the causes of human disorders/differences of sex development (DSD). We mostly intend to isolate and examine tissue from the fetal gonad - most animals bred under this licence will experience no lasting harm and will be killed following breeding. We will attempt, when possible, to target genetic alteration to the developing gonad: a very small minority of mice will be allowed to exhibit abnormalities in the juvenile/adult period caused by genetic alteration. Conditional approaches allow us to overcome lethality and welfare-related issues.

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

The mouse sex determination pathway is highly conserved with humans, in contrast to that of flies, fish or birds. These latter three, for example, lack a sex-determining Y chromosome. We have previously collaborated with a laboratory that uses zebrafish. However, such work is useful only for certain aspects of our research, such as examining whether a mutated gene malfunctions in the context of another species. The use of non-mammalian animals is not sufficient to model human gonad development or biology: this because there are differences in the sex chromosome systems, the organisation of the sex-determination genetic networks and the underlying biology of the gonad, including the role of hormones. However, if there was an opportunity to study a gene with a highly conserved gonadal role in a less sentient animal, we would take it.

The majority of embryos/fetuses studied here will be before 14.5 dpc and therefore before they are protected under ASPA.

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

Any adult mice being administered substances will be handled using best practice techniques. We will strive to restrict harm to the fetal period by, in the vast majority of cases, generating adult animals only carrying a single copy of a defective gene or transgene. Adult animals lacking a functional gene in every body cell will be minimised by the use of conditional gene deletion techniques. No surgery will be performed on this licence.

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

Routes and volumes for administration of substances are taken from LASA guidelines.

The animal house has full AAALAC and ISO9001-2015 accreditation. To conform to these standards we must ensure a high level of quality control on all fronts including husbandry, phenotyping and administrative processes. The ARRIVE guidelines will be followed at all times.

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

I have attended and presented at a variety of 3Rs-related events in the UK, including AWERB Hub events. I have a network of contacts due to my previous participation in bioethics events, my chairing of the local AWERB for 10 years and my membership of an NC3Rs working group. I will implement reduction and refinement through close contact with my colleagues who are striving to do the same in their own model systems, and through regular discussions with NACWOs and animal technicians on site. I also visit the NC3Rs, FRAME and ARRIVE guidelines websites for news updates. Advances that we can implement will be implemented.

5. Identifying genetic and cellular mechanisms of alveolar development and repair

Project duration:

5 years 0 months

Project purpose

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

Key words

lung, alveoli, tissue repair, regeneration, development

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Growing evidence indicates that reactivating or manipulating factors that are important for lung growth during development can be used to repair/regenerate damaged lung alveoli following injury and/or to generate additional alveolar surface area where development has been disrupted. This project will identify potential pro-repair factors that are required for lung development and determine whether they can be manipulated to treat alveolar injury

in adult lungs and/or generate additional alveoli in developing lungs where there is insufficient surface area for normal respiratory function.

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

Why is it important to undertake this work?

The clinical burden of both adult and congenital lung disease (including disease affecting premature babies) worldwide is substantial; in the UK alone, respiratory disease kills one in four people. The lungs have an innate ability to repair themselves, however in many lung diseases, the normal process of tissue repair is disrupted. Examples include Chronic Obstructive Pulmonary Disease (COPD), where alveolar tissue is destroyed and Bronchopulmonary dysplasia (BPD) where insufficient alveoli form in infants born prematurely. There are currently no curative treatments for these diseases, patients are only provided with symptom relief such as oxygen support. Therefore, we will identify factors that are required to generate lung alveoli and investigate whether these factors can be used to help repair or grow additional alveoli and treat these diseases.

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

Outputs from this project will include new information about factors required for lung development and whether these factors can be manipulated to repair lung damage. We may also identify potential treatments to enhance lung repair (which may involve the combination of pro-repair factors with novel delivery techniques designed to optimise pro-repair function). Development of these treatments to clinical trials will be suitable for diseases affecting the alveoli including COPD, Idiopathic Pulmonary Fibrosis (IPF) and BPD and will be outside the scope of this programme of work. Other outputs will involve scientific publications of our findings including methodological papers to enable other groups to adopt novel techniques we have developed.

There will be 3 main outputs from this programme of work:

Comprehensive evaluation of whether at least three mouse models with developmental lung defects also have a role in lung repair and make this data available to scientific community.

Evaluation of whether modification of signalling pathways that affect lung development can modify lung repair using mouse and human models.

Publication of 2 methods papers describing novel models to image and track lung repair that are established during this research.

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

In the short-term (5 years), the outputs will benefit the research community by identifying novel genes required for lung development and/or to repair damaged lung tissue that could be suitable targets for drugs to treat various lung diseases.

In the longer-term, the models, delivery techniques and/or pro-repair molecules that we discover during this programme of work maybe suitable to investigate, as potential treatments for lung diseases. This will be in partnership with pharmaceutical or bioscience companies.

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

We have a network of key collaborators who use similar approaches to us but in different organs including kidney, heart and liver. We regularly share resources and information with these groups.

We publish methodological papers on new techniques and models that we develop to inform the wider scientific community.

We will continue to hold workshops to highlight the uses of precision-cut lung slices to the International scientific community.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

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

In this project, embryonic, juvenile and adult mice will be used. This is because we need to investigate the growth of lungs and lung disease after birth and in adults.

We will also use some genetically modified mouse strains so that we can determine the role of a particular gene at different stages of life e.g. by inactivating the gene in embryos that will then mature into adults or inactivating only once mice reach adulthood.

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

Most typically an animal under this licence will be bred and then killed using a schedule 1 method to obtain tissues (4500 out of 5000 =approximately 90% of all animals).

The remaining 10% of all mice (500 out of 5000) in this licence may be used in protocol 3 to test potential therapeutic interventions for lung injury.

Of the 10 percent of mice that may be used in Protocol 3, the typical type of experiment will be as follows:

To test pro-repair factors for treatment or prevention of lung disease- wild type and mutant mice are administered intranasally (i.n.) with a single substance to induce lung disease or

injury e.g. Influenza or LPS. This is unlikely to cause more than a minimal clinical effect. Following induction of lung disease, a putative pro repair treatment is administered i.n. on up to three separate occasions over a maximum of 14 days, with a minimum of 24 hours in between doses. Some mice will receive a pro- repair treatment without prior induction of lung injury. Mice will be killed by anaesthetic overdose followed by harvesting of blood, tissues and other bodily fluids for further analysis.

Example of the most extreme type of experiment likely in P3- approximately 10% of mice

To test pro-repair factors for treatment or prevention of lung disease- wild type and mutant mice are administered i.n. with a single substance to induce lung disease or injury e.g. Influenza or LPS. This is unlikely to cause more than a minimal clinical effect. Following induction of lung disease, a putative pro repair treatment is administered i.n. on up to three separate occasions over a maximum of 14 days, with a minimum of 24 hours in between doses. Some mice will receive a pro-repair treatment without prior induction of lung injury.

A blood sample may be taken on one occasion and/or in vivo imaging on a maximum of 2 occasions. Urine may also be collected. Under terminal anaesthesia mice may then undergo lung function analysis followed by blood sampling followed by post-mortem harvesting of tissues.

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

None of the procedures used should cause lasting harm. Anaesthetics are used where appropriate to reduce stress for example during administration of substances to the nose. Some of the substances we administer may cause flu-like illness with some loss of appetite and weight however, these symptoms are transient. We will closely monitor mice for signs of ill health such as piloerection, hunched posture, inactivity or inappetence. If any such symptoms are observed, mice will be monitored more frequently and additional measures such as warming or wet mash will be provided. Should the signs persist for a period of 24 hours the animal will be humanely killed. In addition, mice will be weighed daily and any animal that loses 15% or more of its starting body weight will be culled.

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

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

Around 80% of animals will experience mild severity or sub-threshold severity, where animals are bred, genotyped and then killed to obtain tissues and body fluids. Of the 20% of mice remaining 10% used in P3 could reach moderate severity. A further 10% of mice will be used in protocol 2 however, only one quarter of these mice could reach a moderate severity limit i.e. only those where the gene of interest has been completely removed (homozygotes) therefore the total percent of mice that may reach moderate severity is 10% plus 2.5% = 12.5%.

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?

As the lung is a complex organ and the essential respiratory organ of the body, it is not possible to undertake certain comprehensive physiological studies outside the context of an intact organism where all native cell and tissue types are present in their normal numbers and arrangements (e.g. epithelium, mesenchyme, vasculature, lymphatics and nerves). In addition to all these components being present in their normal arrangements (morphology) constituting an entire organ, the lungs must be connected to an active circulatory system and be maintained within a restricted space (thoracic cavity) that is subjected to mechanical cyclic strain as occurs during normal breathing. There is currently no alternative to a whole animal that can reproduce all these factors together.

Another key reason why we cannot currently entirely replace animal use for this project is that this study focuses on how developmental pathways and their disruption have a long-lasting effect on adult health and disease suffered in the postnatal animal. It is therefore necessary to perform these studies on mice with developmental disorders maturing into effected adults. Moreover, we wish to identify genes that are required to establish normal structure of the whole lungs, e.g., for the lungs to form their full complement of airways, alveoli etc.

We do this by analysing the whole lungs both during development and post- natally, if embryonic development is affected, in the context of a mouse carrying a genetic modification in a gene(s) of interest. This type of analysis must be undertaken in whole intact lungs that have developed in a genetically modified in vivo environment and cannot be undertaken in a system such as a lung slice or one that models one compartment of a whole lung e.g. an airway (ALI cultures) or lung on a chip (an alveolus).

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

Whenever possible we make use of non-animal alternatives. Some of our experiments will be carried out using mouse or human cell lines and other experiments are conducted using human lung tissue slices. We also make use of various biobanks of human material e.g. UK Biobank, Northern Finnish

Birth cohort and information for our studies to understand the genetics of lung development and disease. Throughout the project, where possible, non-animal experiments will be employed and we will consult various databases of existing human and mouse information.

Why were they not suitable?

As outlined, it is necessary for us to use animals for some of our experiments so that we can obtain information about how a particular gene or signalling pathway affects an animal at different life stages and how the lungs are affected in the context of 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?

The number of animals is estimated based on previous experience of the number and type of experiments we will do over the duration of the project. Where we had previous data from similar experiments available, online power equation calculators were used to determine animal group sizes required to obtain statistically meaningful data.

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

Where we had previous data from similar experiments available, online power equation calculators were used to determine animal group sizes required to obtain statistically meaningful data. We also employed the NC3R's experimental design assistant where possible to ensure that animal numbers are the minimum required to obtain meaningful data.

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

We will continue to make use of databases of human and mouse information e.g. UK Biobank and Mousephenotype.org to obtain information which will reduce the number of animals we need to use.

Using our experience and that of the animal technicians, we will employ efficient breeding strategies for example, Mouse lines will only be maintained whilst there is a justified use for their continued breeding. Any line with no predicted usage will be cryopreserved and removed from the shelf. Prior to establishment of a colony of GA mice under this licence, any available breeding data will be sought.

Well established breeding calculations will be used to predict output.

Breeding numbers are calculated taking into account average litter size for that stock as well as known neonate mortality. Breeds are set up in a controlled, time restricted manner, to ensure that all mice born are the correct age for the study.

Where breeding information is not known (i.e. for new lines) a small pilot breed will be carried out first to assess viability. These mice, if viable, can be used for the first cohort of

the study, and remaining larger breeds will be set up taking into account information gathered from the pilot breed.

Where possible, we will maximise the data obtained from each cohort of mice. For example, when mice are sacrificed blood, BALF and any relevant tissues will be extracted from the same animals. Where relevant this may include tissues from other organs such as heart, kidney, liver which we will share with our existing collaborators.

Refinement

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

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

The severity of the breeding stock will be held to a minimum by the following strategies: Information gathering- Information on any expected adverse effects will be gathered prior to establishment of a line. Welfare assessment - Systems of comprehensive welfare assessment are in place. These will be used to define the least severe humane endpoints for every GA line. Least severe genotype - Where possible the lines will be maintained as the least severe genotype possible (e.g. heterozygotes will be used for stock maintenance where the homozygous state has undesirable adverse effects).

Sampling techniques - The least severe technique will be used to obtain a biopsy for genotyping i.e. from the ear pinnae phenotyping tests - Lung function analysis will be either in an unrestrained plethysmograph where mice are not anaesthetised and can move freely or under terminal anaesthesia.

Where substances are administered to mice e.g. pathogens, or LPS, we will use the minimum amount of agent, by the least invasive route (typically intranasal administration) which has previously been shown to cause the desired effect.

In many experiments, we will use ex vivo models that we have developed using precision-cut lung slices to address our research questions. For these experiments mice are killed using a schedule 1 method and the lungs are extracted to obtain tissue slices.

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

The mouse is the lowest mammalian species in which the full range of genetic and physiological manipulations necessary for the investigation of lung development and disease can be achieved. It is critical to perform these studies in mammals since there are significant differences between the respiratory systems of frogs and fish to that of humans.

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

Daily monitoring of animals will take place where substances have been administered, this will include monitoring of body weight allows us to define more humane endpoints. Pain management will be provided on the advice of the Veterinary Surgeon for any procedure or situation that requires it. Training is continual making use of new developments and technologies as they become available.

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

The establishment where work under this licence will be undertaken is AAALAC accredited. We will follow published ARRIVE guidelines and as well as information issued by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and the RSPCA.

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

The scientific literature and conferences are sources of information on new developments and technologies, as are workshops run at our establishment on the Replacement, Refinement and Reduction of animal use.

6. Batch Potency and Safety Testing of Foot and Mouth Disease (FMD) vaccine

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

FMD vaccine, Potency, Safety, Antisera

Animal types	Life stages
Cattle	adult

Pigs	juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To ensure that each batch of FMD vaccine is produced is safe and effective for field 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?

To ensure the ongoing protection of the global animal population from Foot and Mouth Disease (FMD) by vaccination. FMD is a highly contagious viral disease of cloven-hooved animals with significant economic impact in cattle, pigs, sheep and goats. In wildlife, all cloven-hooved species are susceptible to FMD with some of them such as African buffalo, acting as a reservoir of the virus without showing clinical signs meaning that eradication of FMD by slaughter is not possible. In susceptible, non- vaccinated domestic farm animal populations, morbidity could be as high as 100% and is an OIE (World Animal Health organisation) notifiable disease.

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

The availability of an effective FMD vaccine is the cornerstone of many national government strategies to prevent FMD outbreaks with their associated animal welfare problems and social and economic and environmental costs.

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

Routine vaccination is used in any countries or zones where the disease is endemic or where there is a risk from circulating virus spreading from neighbouring countries or zones which are infected.

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

N/A

Species and numbers of animals expected to be used

- Cattle: 2350

- Pigs: 2010

Predicted harms

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

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

Protocols 1 and 2. The species, numbers and ages of the animals used is specified in the European Pharmacopoeia. Any deviation from these specifications will lead to rejection of the results by the regulatory authorities.

Protocol 3. The species and number of animals used for this test are determined by the volume and specificity of antiserum required.

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

Protocol 1: Animals will be vaccinated and have their rectal temperature taken for two days prior to vaccination and then for up to fourteen days post vaccination. Animals will be euthanised by a Schedule 1 method.

Protocol 2: Animals will be vaccinated and a blood sample will be taken from superficial blood vessels no more than twice in a 28 day period. Animals will be euthanised by a Schedule 1 method.

Protocol 3: Animals will be vaccinated and a blood sample will be taken from superficial blood vessels (no more than 15% circulating blood volume in a 28 day period or no more than 10% on any one occasion). Animals will be euthanised by a Schedule 1 method.

On the rare occasion for protocol 2 and 3, an additional blood sample may be taken but this will remain within the permitted volumes (no more than 15% circulating blood volume in a 28 day period or no more than 10% on any one occasion)

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

Protocol 1, 2 and 3 - Some animals may experience mild swelling around the injection site. Some animals may experience slight swelling (hematoma) around the blood sampling site.

Animals in protocol 1, may experience a high temperature reading which is outside of the sponsors limits. If this is seen for two successive recordings, the findings are reported to the NVS, who will examine the animal (s) to diagnose if it is the vaccine or an incidental intercurrent disease that is at fault. Treatment or Schedule 1 is dictated by the NVS.

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

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

Protocol 1: Mild – All animals Protocol 2: Mild – All animals Protocol 3: Mild – 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 procedures detailed in this licence application are governed by European and British regulatory requirements which stipulate the methods of testing to be employed.

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?

The number of animals is based on the expected number of vaccines to be tested throughout the life of the project licence.

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

Protocol 1 and 2 – The number of animals used for each test is the minimum number required by the European Pharmacopoeia. Any deviation from these numbers will lead to rejection of the results by the regulators.

Protocol 3 – These numbers would be dictated by the demand from the sponsor.

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

As the number of animals to be used is dictated by the legislative requirements, the only other area of optimisation is to reduce wastage by reusing animals that have completed other projects and where the re-use criteria are fulfilled.

Refinement

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

Which animal models and methods 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 safety test, the European pharmacopeia specifies the most sensitive category of animal to measure any adverse effects of the vaccines on each target species (this usually means the youngest age indicated). The safety test involves the injection of a double dose of the commercial formulation of the vaccine with temperature monitoring.

The original method of assessing vaccine potency was a challenge test with live FMD virus

The European Directive 2001/82/EC indicates that methods other than the challenge method stated in the Pharmacopoeia for determining the potency of FMD vaccine may be used provided that statistical evaluation has established a satisfactory correlation between the two methods. The correlation between the level of circulating neutralising antibodies in animals vaccinated with the batch under test and the level of protection against infection has been well established and all the major FMD institutes and vaccine producers now use the in-vitro assay of neutralising antibody levels as a direct measurement of vaccine potency. This means the test has reduced to usually one injection with commercial formulation of the vaccine and then a blood sample. The European Pharmacopeia specifies cattle, or any other species for which immunogenicity has been shown. There is now an increasing requirement of national governments for the tests to be carried out in at least one of the target species the vaccine is intended for.

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

The pharmacopoeia dictates the species and age of animal required for this regulatory testing based to ensure efficacy and safety of product.

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

All injections and blood sampling are carried out by trained and competent staff and to recognised veterinary standards.

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

Best practice guidance and information is obtained from NC3Rs, IAT, LASA and the RSPCA, particularly. LASA guidelines on administration of substances and taking of blood sample, NC3Rs guidelines on blood sampling. 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?

In addition to staying involved via stakeholders such as LASA, LAVA, NC3Rs, every six months the organisation's 'Species Use and 3R group' meets to discuss all animal work performed at the establishment. A summary of the work performed on this PPL will be presented to the Animal Welfare and Ethical Review Body (AWERB) committee after 18 and 48 months of the PPL. This will include discussions on how improvements on the work can be made.

7. Maternal and neonatal immunity to virus infections

Project duration

5 years 0 months

Project purpose

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

Key words

virus, antibody, infant, vaccine, drug

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 characterise maternal and infant immune responses to virus infection in order to develop novel approaches for control of virus infection in both mothers and offspring.

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

Why is it important to undertake this work?

Childhood virus infections are a major cause of infant mortality. For example, worldwide an estimated 215,000 children under 5 years old die annually from diarrhoea caused by rotavirus infection (Tate et al, 2015), and up to 111,500 children under 5 die from respiratory disease caused by influenza infection (Nair et al, 2011). In addition, the extent of disease in children caused by newly emerging viruses such as COVID-19 is not yet fully known.

In order to protect children from virus infections it is essential we develop highly effective vaccines and anti-viral drugs. We therefore need to understand how the immune system of infants responds to virus infection so that the most effective control strategies can be designed. It is known that the maternal immune response can significantly affect the immune response of offspring, so both groups of individuals must be carefully evaluated together. Using a breeding mouse model of virus infection will enable detailed study of this complicated system.

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

By the end of this project we will have greater understanding of the immune responses of mother and infants to virus infections. We will use this knowledge to develop a new approach to protect babies from one or more of the viral infections of childhood. This may be in the form of a new vaccine strategy, or a new anti-viral drug. These findings will be published in peer-reviewed journals following the ARRIVE 2.0 guidelines (a checklist of recommendations to improve the reporting of research involving animals), and we plan to put forward any promising new products for clinical trials.

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

Improved knowledge of mother and infant immune responses to viruses will be of considerable value to the wider viral immunology research community within the time-frame of this five year project. Development of new vaccines or anti-viral therapies has the potential to reduce childhood disease and deaths from virus infections, although delivery to patients will require lengthy clinical trials which will occur beyond the duration of this project.

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

Partnerships are already established with a collaborator who plans to study new vaccine approaches to virus infection in adult mice. This project will therefore complement ongoing work.

Knowledge gleaned from this project will be published open access in a timely manner in order to disseminate findings as widely as possible. Both successful and unsuccessful approaches will be published and discussed at scientific conferences.

Species and numbers of animals expected to be used

- Mice: 8880

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 study antibodies that are transferred from mother to infant across the placenta and in breast milk ('maternal antibodies'), a mammalian model is required. For this reason, we

have chosen to use the mouse as this is a good and widely accepted mammalian model for this research. This model will enable us to gain understanding of virus infections and maternal antibodies in humans.

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

Female mice will be infected with different viruses to induce protective immune responses. Mice will then be mated in order to study how maternal immunity is transferred to their offspring. Immune responses in both mothers and pups will be assessed by collecting small volumes of blood from the animals during life, as well as extensive study of their organs after the animals are killed at the end of each experiment.

Pups may be infected with virus within the first week of life, or later as adults. Virus infection levels will be measured either by studying stool/blood samples, imaging mice under anaesthesia, or from organ analysis after death.

New vaccine approaches will be tested by either vaccinating the mother before/during pregnancy, or by vaccinating young pups. To test how effective vaccines are, pups will be infected with the viruses when adults.

New anti-viral drugs will also be tested in mouse mothers and pups to see if new ways of treating virus infections can be identified.

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

Some of the viruses studied may cause mice to lose weight in the first two days after infection, but weight normally returns back to normal within approximately 7 days. Some of the viruses used may also cause signs such as ruffled fur and rapid breathing during the same period. All mice that show these signs will either be killed if signs reach predetermined humane endpoints such as weight loss of 15% plus clinical signs of disease, or if the mice have not returned to full weight within 2 weeks.

Although mice infected with some viruses may develop signs of disease at high doses of virus, this project aims to identify improved vaccination and treatment options. It is therefore anticipated that only the control mice which don't receive the vaccine or treatment in each experiment are at risk of experiencing disease. Furthermore, careful calculation of virus doses will be performed in preliminary experiments to ensure that disease is kept to a minimum.

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

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

Mice: mild severity 90%

Mice: moderate severity 10%

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 use of animals is essential to study the overall immune responses to virus infections. This is because the immune response to viruses is very complex and cannot be replicated in cells in a laboratory. Furthermore, to determine how effective new vaccine approaches are, experimental infections in mice are required.

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

We have considered, and will initially use cells in the laboratory for all preliminary experiments. This is because different cells can readily be infected with the viruses we are studying, and this will allow us to study the effects of viruses and antibodies on a single cell level.

Why were they not suitable?

Experiments in the laboratory will be suitable for first testing whether virus replication can be blocked by specific antibodies or drugs. However, to study complex immune responses that involve many different types of cell working together, infections and vaccinations of live animals are essential.

Reduction

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

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

We have extensive previous experience of infecting mice with viruses, which enables us to make good estimates of how many animals will be required for each experiment. In addition, we have used a specialised programme called the NC3Rs Experimental Design Assistant to help us calculate the number of mice needed.

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

Extensive review of the scientific literature has been conducted to enable predictions for likely outcomes of experiments planned (e.g. suitable virus doses, efficacy of maternal antibody transfer), so the minimum number of mice can be used. I have also sought the advice of more experienced colleagues to ensure animal numbers are at a minimum and welfare is maximised.

We will test all new medicines for treating virus infection in cells in the laboratory first, and only progress to animal experiments if positive results are obtained.

Where possible, we will infect mice with viruses that can be tracked using specialised imaging equipment. This will allow us to monitor how much virus is present in each animal by imaging, instead of having to kill the mice to measure the amount of virus in each organ.

The PREPARE guidelines have also been consulted for formulation of this project, and these will be followed to ensure continued communication between the animal facility and our team.

We have used the NC3Rs Experimental Design Assistant to help us calculate the number of mice needed, and will continue to use this 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?

Breeding programmes of genetically altered mice will be optimised to ensure as little over-breeding as possible. This project will aim to keep “surplus” animals to a minimum. In order to reduce the numbers of breeding pairs the mice will be kept as purebred lines (when appropriate), provided that they do not show any signs of disease.

Samples collected from any mice as part of experiments planned will be stored long term at -20C. This will make the samples available for future analysis by scientists working on this project, and also for any collaborators.

Refinement

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

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

This project uses mice that will be infected with different viruses, then their immune responses will be studied by collection of blood samples, and analysis of different organs after death.

The mouse has been selected for this project as it is the best way to study human anti-viral immune responses. The mouse also benefits from well-established and robust technologies for modifying their genes. Where possible we will use experiments of the shortest duration so long as to do so will yield satisfactory data.

In order to understand how maternal immunity impacts on infant immunity to virus infections, we must work with viruses that can replicate in mice. These can cause clinical signs at high doses, but is essential that we follow the outcome of infection to fully investigate immune responses that can lead to viral clearance. If we kill animals at the first sign of infection we cannot investigate what responses are required that allow them to clear the infection naturally. Previous experience has shown that weight loss can correlate closely with the amount of virus in a mouse. Monitoring weight loss is therefore an effective way of determining infection, and is a widely used and accepted measure. However, weight loss is not the only measure of infection and we will use a comprehensive monitoring and scoring system to assess the animals throughout experiments.

Finally, this project aims to use non-surgical embryo transfer as a new approach for breeding genetically modified mice. This method will be explored and optimised over the course of this licence as a refinement to the existing surgical method of embryo transfer.

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

Study of how maternal immune responses impact on the immunity of infants requires use of animals that transfer antibodies to their offspring via the placenta or in milk. This specialised method of antibody transfer therefore means species such as fish, flies or nematodes cannot be used for this project.

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

All mice that are infected with viruses will be monitored twice daily for any clinical signs of illness (e.g. ruffled fur and weight loss in adult mice, or rejection by the mother in pups). The results of these will be recorded in a spreadsheet for each experiment. Additional mash will be provided for sick animals unable to access the food hopper, and extra nesting material will be given to improve the comfort and warmth of virally infected mice.

Weights for all animals over 3 weeks of age will be recorded once daily after infection until weights have returned to normal. Mice will thereafter be weighed once weekly. Again, a record of weights will be kept.

To assess levels of virus we will use minimally invasive sampling, such as sampling from faeces, or imaging where possible.

For any procedures requiring anaesthesia, mice will be monitored closely for the duration of their recovery.

For viruses given by mouth, a drop of sugar solution will be placed on the end of the delivery tube in order to encourage swallowing.

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

We will use guidelines from the Laboratory Animal Science Association (LASA) to make sure all experiments are conducted appropriately. In particular we will follow the 'Guiding principles on good practice for Animal Welfare and Ethical Review Bodies'.

To ensure refined experimental design we will follow the PREPARE guidelines for planning experiments, and for thorough, responsible reporting of results we will follow the ARRIVE guidelines.

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

We will continue to be informed about the 3Rs during the duration of this project by regularly checking our institute's 3R's search page, and being registered for the regular NC3Rs e-mails and newsletter updates. Regular reference to guidance documents provided by Laboratory Animal Science Association (LASA) and the RSPCA will be made.

We will also ensure continued contact with the organisational teams in the facilities in which our mouse work is conducted. Any new recommendations will be incorporated into our experimental plan wherever possible.

8. Regulation and development of adaptive immune responses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, Antibodies, Infection, Vaccines, B cells

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project to understand the cellular and molecular mechanisms that control adaptive immune responses, with a focus on how antibody-mediated immunity develops.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit – these could be short-term benefits within 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 most important medical interventions available, saving millions of lives each year.

Vaccines work by “educating” the immune system about what potential pathogens “look like”, i.e. by inducing immunological memory against them. An important way that this is achieved is by stimulating the generation of pathogen-specific antibodies. Antibodies are soluble proteins that are found in blood and in tissue fluids and are made by terminally differentiated B cells, a type of white blood cell. The most potent antibodies usually have “neutralizing” capacity, that is they can tightly bind (i.e. with high affinity) key sites (epitopes) that invading microbes use during the infection process. For example, effective influenza vaccines stimulate the production of antibodies that recognize and block the function of hemagglutinin, a glycoprotein that the virus uses to enter lung cells.

The goal of our research is to understand how B cells “decide” which antibodies to make during immune responses. B cells face an enormous challenge, because they need to be able to recognize an almost infinite number of target epitopes. Furthermore, microbes commonly change (or disguise) the proteins that they express to avoid and escape immune recognition. Fortunately, antibody responses are also highly plastic: B cells can edit their antibody encoding genes and therefore engineer bespoke reagents for each pathogen that they encounter. This remarkable feat is achieved in specialized structures known as germinal centres, where B cells mutate their antibody genes and then select the best variants. Our research is focused on understanding the development and regulation of adaptive immunity in general, but we have a major focus on determining how particular antibodies are selected in germinal centres.

Despite effective and safe vaccines existing for many important diseases, there are several common and devastating diseases against which it is still not possible to immunise. Among these are HIV/AIDS (caused by human immunodeficiency virus, HIV) and malaria (caused by Plasmodium spp), which still kill many hundreds of thousands of people each year. The development of vaccines that induce long- term immunity against Covid-19 will also benefit from a better understanding of how antibody responses are orchestrated. Consequently, there is an urgent need to learn more about how immunological memory is established and regulated, and then to use this knowledge for informing vaccine development strategies. By learning what determines antibody specificity during immune responses, we hope to gain a better understanding of what is needed to drive protective immunity.

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

This is a basic science project that is expected to lead to the generation of new fundamental knowledge regarding how antibody dependent immune responses develop and are regulated. We hope to identify new mechanisms that can be exploited in the development of vaccines.

We aim to identify biochemical pathways that can be targeted in diseases that involve dysfunctional B cell responses, e.g. lymphomas and autoimmunity. We anticipate contributing to a generally improved understanding of how adaptive immunity is established. The discoveries made through this research will be reported in peer reviewed publications.

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

We aim to decipher the mechanisms determining the magnitude, quality and nature of antibody- dependent immune responses in settings of infection and after immunisations. Antibodies are soluble proteins that are made by the immune system that specifically bind to particular foreign antigens (sites on pathogens), and by doing this they either neutralize the pathogen by inhibiting its normal functions or induce its destruction. Almost all currently approved vaccines, as well as many of those in development, are thought to rely upon antibodies for their efficacy.

The first beneficiaries of our research will be other immunologists in academia and in private industry (benefits expected to be evident within 2-5 years). Our aim is to decipher some of the key principles underpinning the regulation of antibody responses, therefore the findings are likely to be of interest to broader audiences beyond those in our immediate field. Some of our earlier findings are reported in popular textbooks used to educate medical and science students around the world, and we anticipate a similar impact again (a second beneficiary, expected in 4-10 years). Science is a highly iterative process, therefore major findings help inform the next research steps to be taken by multiple groups (impact over longer periods of 5-10 years). Beyond the findings themselves, the development of new approaches and techniques is also likely impact other fields such as T cell biology and developmental biology (second beneficiary, benefits in 2-5 years).

Inappropriately targeted antibody responses are responsible for a range of autoimmune diseases. As such, the third beneficiary will be scientists and clinicians involved in researching or treating autoimmune diseases (third beneficiary, 2-5 years). By determining the mechanisms controlling normal antibody-mediated immune responses, we anticipate identifying pathways that might be dysregulated in disease. Similar principles are true for some cancers also; dysregulated germinal centre responses can cause lymphomagenesis (fourth beneficiary, 3-6 years). The indirect impact on understanding disease pathogenesis is likely to take a further 3-7 years.

Vaccine development involves making reagents that initiate antibody responses against targets of interest, i.e. particular sites on pathogens. For example, the most promising approaches currently under development for vaccination against human immunodeficiency virus (HIV) involve trying to very carefully shepherd antibodies down defined developmental pathways in germinal centres.

While such pathways only occur occasionally in the course of natural infections, it is thought we should be able to manipulate immune responses using vaccines in such a way as to induce them.

Effective vaccines will also depend upon stimulating the generation of sufficient antibody titres (i.e. high concentrations of antibodies in the blood). Our research is aimed at understanding how antibody maturation pathway trajectories are determined, and what controls the generation of antibody secreting plasma cells, therefore our findings will benefit researchers working in this area (fifth beneficiary, 2-5 years).

In summary, our results will directly benefit basic science in the short and long-term and expect to indirectly contribute to the understanding of various diseases/treatments.

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

Output will principally be in the form of peer review publications. We always publish our findings in well-respected international journals, thereby ensuring optimal distribution. Our institute has a dedicated public engagement officer who will help draw attention to studies as and when they are released (e.g. through social media and traditional press platforms). Beyond publications, we will present our findings at conferences at the earliest appropriate times, to ensure quick dissemination of newly gained knowledge. Where appropriate, we will collaborate with other research groups in related disease areas so that they can quickly make use of our discoveries, tools and approaches.

Species and numbers of animals expected to be used

- Mice: 34250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 to research the biology of adaptive immune responses. Adult mice are used because they have fully formed immune systems that are similar to that of humans.

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

Genetically altered or wild-type mice may receive immunisations to induce immune responses. In some cases, they may also have immune cells from other mice transferred into them using injections.

Alternatively, mice may receive bone marrow transplants following whole body irradiation. At certain stages of immune responses, mice may receive injections of certain drugs, such as those used in people, to block certain molecular pathways. By subsequently comparing treated and control mice, we will learn how certain molecular pathways impact immune responses. The measurements made may include asking whether mice are better or worse protected from a certain viral infection (e.g. Influenza A virus), or we may harvest lymph nodes from euthanised mice and examine the quality of the immune response.

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

The expected impacts will be similar to those experienced by humans receiving similar treatments. Where mice receive whole body irradiation and bone marrow transplantation, they are likely to feel unwell for some period and will be susceptible to opportunistic infections. Such mice will be

housed in appropriate controlled conditions to limit the chances of infections and may receive prophylactic antibiotic treatments. Immunisations may cause some local

irritation/inflammation and can be associated with a fever-like response. The side effects of drug treatments will depend upon the specific compound but like in humans they can range from no undesirable effects to moderate levels of systemic or local discomfort. Influenza and malaria infections are usually self-resolving but do cause general periods of feeling unwell that can last multiple days and cause mice to reduce their food and/or water intake.

Expected severity categories and the 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 (>80%) will suffer subthreshold or mild severities of suffering. Some mice (<20%) are expected to suffer moderate severities of suffering.

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

- Killed

Replacement

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

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

We aim to understand how protective antibodies develop in germinal centres. Germinal centre responses are a complex multi-cellular and multi-lineage process that cannot be recapitulated in vitro. Germinal centre B cells cannot be isolated from human blood samples. Germinal centre B cells are programmed to die or differentiate outside their native environment, further limiting the types of ex vivo experiments that can be performed. For these reasons, it will not be possible to determine the mechanisms controlling antibody development without the use of animal experimentation.

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

- We have considered using human blood samples.
- We have considered using human tissue samples (e.g. tonsils).
- We have tried using in vitro differentiated “induced germinal centre B cells” (iGCB cells). In silico modelling.

Why were they not suitable?

Human blood samples do not contain germinal centre B cells or any of the other cell types found in germinal centres. Antibodies do not undergo affinity maturation in the blood.

Human tissue samples. These are useful for descriptive and comparative purposes, however it is not possible to probe specific mechanisms this way.

In vitro differentiated “induced germinal centre B cells”. iGCB cells share some properties with real germinal centre B cells, but in reality they are more different than similar.

We collaborate with in silico modellers, however while such approaches are useful for prediction, they cannot test real biological mechanisms or functions.

Reduction

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

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

Numbers of mice have been estimated based upon typical numbers of experiments required to test specific hypotheses. These estimates have taken into account our experience from the last 16 years as well and projected changes in project scope during the next five years. Average animal group sizes take into account POWER calculations for reaching statistical robustness and prior experience of levels of variability within groups.

Numbers of animals required for breeding protocols take into account animals generated with the wrong genotype as well as animals that are required for colony maintenance but will not be moved to other protocols. Animal numbers listed in experimental protocols include those transferred from breeding protocols as well as those purchased from the core facility and outside vendors.

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

The number of excess mice generated through breeding will be minimised by constant and careful monitoring of breeding programmes. We will endeavour to repurpose mice that are identified as having the wrong genotype for their initial purpose (e.g. using them in place of wildtype animals in experiments where appropriate, or for parasite production).

Careful experimental design, including efforts to increase uniformity and reduce random variation, will be used to detect significant differences with low numbers of animals. Blinding is introduced where possible, for example in scoring histology. Therefore, our approach is to use factorial designs and blocking to remove nuisance variables, if numbers allow. The majority of our experiments produce quantitative data that can be analysed by parametric methods such as ANOVA or t-test (STATA, www.stata.com). Experimental procedures are reviewed within our group and at institutional inter-group meetings. The university biomedical services unit and training support officer provide statistical support.

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

The number of animals used will be reduced by careful animal breeding strategies. Examples of careful breeding employed will include advanced experimental planning so that animals are bred in greater numbers for specific experiments where they are needed, and care will be taken to ensure that required animal numbers are generated concurrently so as to allow good experimental group sizes. In cases where single mice are required to carry multiple genetically altered alleles, breeding strategies will be carefully designed to ensure efficient inheritance of the desired genotype; for example by breeding 69omozygous mice together rather than heterozygous mice (potentially reducing animal use two-fold relative to heterozygous breeding strategies).

We have an active collaboration with a computer modelling group and, where appropriate, in silico predictions will be used to target experimental approaches more efficiently.

We will share malaria parasite stocks with our collaborators to reduce the number of animals required for their generation. When using new experimental approaches, small pilot experiments will be used initially to assess feasibility and their appropriateness.

Refinement

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

Which animal models and methods 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 breeding, maintenance and use of genetically altered animals. In the majority of cases, the genetic alteration itself will not cause the animal any harm. In a minority of cases, animals harbouring gene alterations that cause them to be immunosuppressed are required. Such modifications include the interruption of genes that are required for the development of certain immune cell types. We will always use models that are the least harmful while achieving the required effect; this will often involve using genetic alterations that are least likely to cause mice to suffer infections from opportunistic pathogens.

Our research is aimed at understanding how immune responses are regulated. For this, we commonly need to infect or immunise mice with pathogens or vaccines, respectively. Vaccines usually cause less harm than infections, therefore these will always be our preferred choice. Vaccines usually include adjuvants that boost their immunogenicity and promote longer-lived immune responses. A wide variety of adjuvants exist and each differs in terms of their inflammatory properties, and therefore how likely they are to cause undesired effects (such as local inflammation, transient fever). Where possible, we will use the least harmful adjuvant type (e.g. alum) to minimise the harm caused, and we will also always chose the least harmful appropriate route of administration (e.g. often using subcutaneous administration rather than intraperitoneal).

We will avoid the use of Complete Freund's adjuvant wherever possible – we expect using in very rarely (in <1% of immunization experiments). These experiments sometimes require us to also transfer immune cells from one mouse to another, however the use of best practice techniques means that this rarely causes more than transient mild discomfort to the mouse.

Immune responses to vaccines and infections are not identical, and therefore we will sometimes need to use pathogen challenge models (e.g. influenza A virus, malaria causing plasmodium parasites, vaccinia virus). For such experiments, we will use the least harmful strains possible, and use appropriate inoculum doses that are less likely to cause significant disease. This will minimise the severity of symptoms caused by the infections. Following infection, mice will be carefully monitored for signs of suffering and experiments will be terminated if animals approach the severity limits outlined herein.

To decipher the molecular and cellular events controlling immune responses, we will sometimes treat mice with defined drugs or pathway altering substances. To minimise harm, we will carefully determine, based upon the scientific literature, an appropriate dose regime. Where the expected effects of any drugs on the animal's welfare are not known, or where harmful effects are likely, animals will be carefully monitored for signs of suffering. If repeated injections are required, we will consider whether the installation of osmotic pumps for continual slow-release administration.

Bone marrow chimeric mice are generated by treating animals with a dose of irradiation that ablates their existing immune system, followed by reconstitution the irradiated mice with donor bone marrow. This experimental approach allows us to assess whether certain genes are required in certain cell types. To minimise the harm of the whole-body irradiation, mice will usually be treated with a course of antibiotics for approximately 4 weeks following the treatment, thereby minimising the likelihood of them suffering opportunistic infections. The minimal irradiation dose possible will be used, and irradiation doses will be split to limit harmful effects to other tissues and organs. We will sometimes use approaches that involve adoptive transfer of mature immune cells into non-irradiated hosts to avoid using irradiation-based approaches.

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

Mice are the least sentient animals that have immune systems similar to that of humans. We need to use adult mice in order to mimic closely the adult human immune response, and similar experiments cannot be performed in less sentient animals due to them lacking a similarly complex immune system. Immune responses take days to fully develop and can continue evolving for several weeks or even months. For this reason, terminally anaesthetised animals cannot be used in most experiments.

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

We will continue discussions with vets and animal technicians regarding how our protocols can be refined further. We continue to monitor the scientific literature for approaches that are more efficient and less harmful, for example new adjuvants for immunisation. One

example where this has already proved useful is in identifying technological advances that permit in situ photoconversion through the skin (where previously surgery was required). Another example is the use of hock injections in place of foot- pad injections. We also will utilize techniques for silencing/expressing (e.g. Crispr) gene that involve retroviral/lentiviral delivery platforms as an alternative to generating new genetically altered mouse lines.

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

We will use the 3R resources (https://www.nc3rs.org.uk/3rs-resources).

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

All lab members will regularly attend the animal facility users meetings to learn about the latest developments in the 3Rs.

We will continue discussing our experimental approaches with facility NACWOs and NVSs so that protocols can be constantly improved. We will regularly visit the 3R website (https://www.nc3rs.org.uk/the-3rs) to obtain the latest updates.

9. Molecular basis of meiotic recombination and its impact on fertility

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

fertility, chromosome pairing, cell division, egg, sperm

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?

This project aims to understand how maternal and paternal chromosomes - the DNA structures that carry the genetic information in every cell of the body - pair and exchange material (recombine) during the cell division that produces eggs and sperm in mammals (meiosis), and to determine the impact of this process on fertility.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 cell division that produces eggs and sperm (called meiosis) is fundamental for healthy sexual reproduction in mammals. In sex cells, maternal and paternal chromosomes must pair and exchange genetic material (recombine) to maintain a correct number while dividing, which ensures successful meiosis and normal fertility. However the mechanisms

that control this process remain poorly understood. This project will characterise in detail existing and novel regulators of each of these steps to understand how they impact meiosis and fertility levels in the mouse.

Errors in meiosis result in miscarriage, birth defects, and infertility in humans. Infertility affects 1 in 6 couples worldwide. Despite recent advances in the treatment of male infertility in particular, the exacts defects involved remain largely unknown. The results from this project in mice will be directly relevant to human fertility, and indeed infertility. Findings may therefore assist diagnosis and counselling in patients with fertility issues, and open new treatment options.

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

This project will greatly advance our knowledge and understanding of the molecules involved in meiosis (the cell division that generates sperm and eggs), and how they impact fertility in mammals. These experiments conducted in the mouse will provide a detailed characterisation of the role of both known (but poorly understood), and novel (yet to be discovered) protein factors that regulate this fundamental process.

We expect the findings will be published in high ranking scientific journals, and will support successful applications for further funding. All new computer-based methodologies developed, as well as the data generated from the animals used during the course of this project will be extremely valuable and become available to the wider scientific community for use in other types of analysis that may advance further research in this field.

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

As meiosis (the cell division that produces eggs and sperm) is highly conserved across mammals, we anticipate the findings will have an immediate impact and advance both scientific and medical research into human fertility.

In the long term, the molecules identified and characterised in these studies in mice will provide new fertility candidate genes for screening which may help diagnosis and counselling in couples with fertility issues, and open new treatment options for patients with infertility.

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

This project is established in close collaboration with three other groups locally, each bringing specific skills and expertise. This enables an efficient and coordinated analysis of the mice studied, and the integration of the findings into a highly informative view not otherwise possible. The experimental strategies will be continually reviewed, and revised if necessary against the objectives, as a collaborative discussion. The findings from this work will be published (including all data generated made publicly available), and regularly presented at relevant conferences in the field, providing additional collaborative opportunities that may advance further the progress of the scientific discoveries.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

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

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

Meiosis, the specialised cell division that produces sperm and eggs, occurs exclusively in cells of the male and female reproductive tissues, respectively. To characterise the protein factors involved, and study their role in maintaining normal fertility in mammals, we chose the mouse as our experimental animal model.

The laboratory mouse is the most widely used animal model in research to study key biological processes in human health and disease. Due to similar genetics and biology between mice and humans, genetic manipulation in the mouse can be used to identify disease mechanisms and develop treatments for humans. Importantly, infertility naturally occurs in the male offspring of crosses between certain mouse species; these represent natural (non genetically modified) fertility models to study. In addition, sites where genetic material was swapped between maternal and paternal chromosomes (the structures that carry the genetic information in all cells of the body) are easy to detect in these crosses. Finally, a number of mouse mutants already exist for key molecules involved in meiosis; this provides a great resource for this project. For all these reasons combined, the mouse represents the most appropriate model for this research and its relevance to infertility in humans.

Meiosis occurs at specific times in males and females during their lives. In females it begins in the mother's womb, in the ovary of the foetus, and pauses at birth, resuming later at puberty to complete at the time of sperm fertilisation; in males it starts in the testis around 10 days of age, and carries on into adulthood. We will therefore use mice ranging from foetal to adult stages.

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

The animals will only be used for breeding and maintenance of the lines of interest to:

assess fertility in live animals (through breeding) provide reproductive tissues for downstream analysis of meiosis

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

The genetic modifications engineered during this project are not expected to cause any harm to the animals besides a reduction in fertility (ranging from a partial to a complete 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)?

Mild severity: 100% of all mice

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

- Killed
- Used in other projects

Replacement

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

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

Meiosis is a cell division that occurs exclusively in sex cells of the male and female reproductive systems. While we will be using non animal, easy to expand, cancer cells to study certain properties of the molecules involved in this process, these systems lack the specialised cell environment only present in sex cells. It is extremely difficult, however, to maintain sex cells alive and functional outside the animal to carry out experiments. At present, meiosis can therefore only be studied in the whole organism by analysing reproductive tissues of animals from relevant strains. In addition, this project requires live animals to investigate the impact on fertility of certain genetic modifications, by monitoring breeding performance to quantify fertility levels.

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

Cancer cell systems: before moving to animal studies, if appropriate, we will first test the relevance of molecules of interest in cancer cell systems where they can be artificially expressed and some aspects of their function studied. In addition, such system has the advantage of high expression (in contrast to typically very low levels in the living animal), and more complex, quicker gene manipulation than possible in the mouse. The findings from these studies can then guide and complement experiments in animals, directly impacting animal numbers down. In some instances, they may even preclude animal strain development if the function of the molecules tested do not prove to be relevant.

Sex cell systems: a number of studies have demonstrated the benefit of using mouse embryonic (stem) cells to characterise some aspects of meiosis, and we are keen to try and implement these more relevant cell systems for functional studies in the future. Recently, this cell type was successfully turned into sex cells using a specialised cocktail of factors. If feasible, we would consider implementing this methodology in the future to replace some of the animal-based studies. This alternative, however, is likely to be technically challenging with limited success. This would have to be weighed against the level of replacement and reduction in animal use this approach would potentially enable so as not to compromise the progress of the research. Trials would first be required to determine the feasibility of this methodology in our hands.

Why were they not suitable?

We have successfully used in the past and intend to continue to use as much as possible non animal cancer cell systems where key molecules can be artificially expressed and some aspects of their function studied. However, as mentioned earlier, these systems lack the specialised factors only expressed in the male and female reproductive tissues, and cannot fully replace (only contribute to a reduction in animal numbers) the studies in the whole body where the correct cell environment is present.

Reduction

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

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

Estimates are primarily based on the actual number of animals used on 2 previous Licences with similar animal experiments, taking into consideration:

established mouse lines of interest already breeding on the current Licence

new genetically altered mouse lines for genes of interest that will be generated during the course of the project

On average, we used 1000 mice per year over 5 years for maintenance of mouse colonies and experimental animals, all lines combined (average of 5 lines being studied). Based on an average of 8 lines in breeding, we estimate we will use a total of 8000 mice over 5 years to complete this project.

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

Wherever possible, if appropriate, the molecules of interest will be tested first in cancer cell systems before moving to animal studies. We have successfully used and will continue to use cancer cell lines wherever possible to help inform and refine the animal studies, therefore working towards a reduction of the number of animals needed for experimentation. However, these cell systems lack the specialised environment only present in sex cells, and therefore remain limited for functional studies.

During the experimental design, we searched and will continually review available cryobanks and databases, including MGI (http://www.informatics.jax.org), the international mouse knockout consortium (http://www.knockoutmouse.org), and the DNA Archive for ENU chemical mutagenesis screens (http://www.har.mrc.ac.uk/services/archiving-distribution/enu-dna-archive) for existing mouse lines of relevant gene mutants and fertility models. Every care will be taken to avoid duplicating animal resources.

All new analysis downstream of animal tissue collection will be first trialled and optimised in pilot experiments on a small group of animals to estimate the minimum number to be used to achieve the results.

Once determined, we will carefully control the breeding to avoid excessive and unnecessary production 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?

Breeding: breeding of all colonies will be reviewed every 2 weeks with the animal care staff to produce the numbers required and, if possible, only the genetic modification desired, avoiding excessive and unnecessary production of animals.

Sharing of tissues: we will aim to share the tissues from the same animal between multiple analyses. When new opportunities arise, pilot experiments will be conducted to assess the feasibility of these approaches.

Refinement

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

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

The mouse is the most widely used animal model in research to study key processes in human health and disease. Due to similar genetics and biology between mice and humans, genetic manipulation in the mouse can be used to identify disease mechanisms and develop treatments for humans.

Based on these principles, we have chosen to use the mouse as our experimental model to study meiosis (the cell division that produces eggs and sperm), and its role in fertility in mammals.

Importantly, infertility naturally occurs in the male offspring between very distinct mouse species, providing natural models of infertility. In these, and in other fertile crosses between distinct mouse strains, sites where some genetic material was swapped between maternal and paternal chromosomes (the structures that carry the genetic information in all cells of the body) are easy to detect. These mice are therefore very informative strains in which to introduce genetic modifications of interest to test their impact on meiosis and fertility.

During this project, we will characterise existing and newly generated mouse mutants for key genes of interest expressed in reproductive tissues. No harmful consequence is

expected from the genetic modifications studied in these animals besides an impact on fertility (ranging from a partial to a complete loss). This project only requires breeding and maintenance of these mouse models to assess fertility in live animals, and provide reproductive tissues for analysis of:

fertility levels by measuring the weight of testes and ovaries, and counting eggs and sperm

pairing of chromosomes by antibody staining of sex cells

binding of key molecules on chromosomes

changes in expression of key genes during meiosis

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

The mouse is the most appropriate animal model to study the cell division that generates eggs and sperm, and characterise the key molecules involved in this process for the reasons outlined above. Dictated by the biology of the process we are studying, we will use mice ranging from foetal to adult age. Like in humans, this cell division occurs at specific times during the development of male and female mice: in females it begins in the foetal ovary and arrests at birth, resuming later at puberty to complete during egg fertilisation by sperm; in males it starts in the testis around 10 days of age, and carries on into adulthood.

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

No harmful consequence is expected from the genetic modifications introduced in these animals besides an impact on fertility (ranging from partial to complete loss).

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

We will follow the published guidelines for best practice "Refinement and reduction in the production of genetically modified mice, Laboratory Animals Vol 37, Supp 1, July 2003".

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

The licence holder and all animal users involved in the project are regularly updated on advances in the 3Rs via the following routes:

termly 3Rs newsletters published by the 3Rs sub-committee of the ACER (Animal Care and Ethical Review) Committee at the University are circulated to all animal users: they provide an update on the 3Rs advances in many areas of research and give specific examples of good practice and successful implementation in specific areas of research across the University. Useful links to other 3Rs resources and upcoming 3Rs workshop events (both internal and external) are also advertised here.

Animal Welfare Meetings for animal facility users (3 times/year): the Regional Programme

Manager for the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) and NACWO (Named Animal Care and Welfare Officer), are presenting examples of successful trials and new implementations of the 3Rs from projects within the University (also published in the 3Rs newsletter). Useful links to 3Rs resources and upcoming 3Rs workshop events (both internal and external) are also circulated during these meetings.

discussion with the Regional Programme Manager for the NC3Rs: opportunities for the implementation of a recent advance in the 3Rs within or outside the University, or the development of a novel approach can be explored in more detail.

N3CR website: www.nc3rs.org.uk.

10. Understanding the Mechanisms of Healthy and Unhealthy Ageing in the Central Nervous System

Project duration

5 years 0 months

Project purpose

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

Key words

Ageing, Neurodegeneration, Synapse, Calcium homeostasis, Memory

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to understand the mechanisms that lead to changes in synaptic and network properties of excitable cells within the brain that accompany natural ageing processes and neurodegenerative conditions such as Alzheimer's disease.

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

Why is it important to undertake this work?

The average age of the general population is increasing but a lengthened life expectancy is often accompanied by a lengthened period of poor health. Understanding how we can age naturally but well will help us to improve the quality of life of the ageing population and help to reduce the societal impact that ageing has. Whilst many people age naturally relatively well, others suffer from neurodegenerative diseases that not only reduce life expectancy but seriously affect the quality of life. Alzheimer's disease is the most common neurodegenerative disease in the western world. It not only shortens life span and it has a debilitating effect on the sufferer, it has a huge impact on the families and carers of the victim as well as on society. Understanding how the brain ages naturally compared to the effects of agerelated neurodegenerative diseases will help us to improve the quality of life of older people as they age and help to establish therapeutic approaches to reversing both healthy and unhealthy affects of ageing.

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

Information

Our overall objective is to establish how age-related changes in synaptic properties affect neuronal activity and the way in which animals learn. By understanding this, we will provide the scientific community with knowledge that will enable us to develop therapeutic strategies to slow down and possibly reverse the cognitive effects of ageing. Alongside this, we will use similar approaches to better understand what changes occur during the early phases of neurodegeneration at a point where neuronal damage may be limited providing some opportunity for treatment. We expect that the work that derives from this project will help us and others gain an insight into these natural and unnatural effects of ageing.

Publications

We hope that each of the 4 objectives of this project will bring one or more publications. Publications will be based around first the synaptic effects that we discovery. For example, we will plan a paper that examines the effects of endocannabinoids on pre- and post-synaptic properties in the hippocampus and a second paper examining their roles in the cerebellum. We will then establish how neuronal activity is affected during learning and then complete the circle by testing whether chronic treatment with endocannabinoids reverses the cogntitive decine associated with aging. In parallel, we will test to see how

beta amyloid deposition in APP knock in mice affects synaptic signalling and then neuronal activity and behaviour.

Products

My laboratory has a track record of developing new technologies. We have 3 patents for microscope development and we were awarded a grant recently to develop a super-resolution miniature microscope. We will use this microscope as part of this project to visualise neuronal activity at high spatial and temporal resolution. There is the possibility that this may become a commercial product that we can provide to other researchers.

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

We expect that the work that derives from this project will help us and others gain an insight into the natural and unnatural effects of ageing. We will publish as much of this work as promptly as we can but the timescale will depend upon securing appropriate funding, completing the work and writing it up. We expect that much of it will be published during the lifetime of the project but it is very likely that publication will continue beyond the project duration.

The neuroscience community will benefit from this work because the results will add to the overall knowledge that we currently have on the processes of ageing. Despite age-related illness being a major societal problem, relatively little is known about the ageing mechanism. In particular it is important for us to understand how we can improve the quality of life in a society where life span is increasing but the last few years of life are often difficult for both the elderly person and for their carers. Whilst it is optimistic to think that we will find a 'cure' for ageing over the lifetime of this project, we certainly expect to establish potential approaches to slow down the effects of ageing.

Beneficiaries will also include the undergraduate and post-graduate students and post-doctoral researchers who will participate in this project. They will obtain training in in vitro and in vivo techniques, learn new methods and gain an understanding of the physiology of ageing and neurodegenerative diseases.

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

We will publish as much of this work as promptly as we can. Two approaches will be made. The first will be based on the scientific outcomes of the research and will follow the usual approaches. Since we are involved in technological development, we will also be able to combine some of the results with more technical papers that describe the methodology of the work. This will encourage others to adopt similar strategies. Alongside standard publications, we will continue to attend conferences and liaise with colleagues to help share and adopt best practice.

Our transgenic mice are used by several other groups as a tool to better understand the role of presynaptic processes. We have started collaborations with groups who work on tinnitus and hearing and overseas groups working on mechanisms of Alzheimer's disease. I hope to deposit mice into a suitable repository so that others will be able to use them.

As we develop new technologies to help complete this work, we will endeavour to make these technologies available to others. This may be through an open technology model but could also include a commercial approach.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 120

Predicted harms

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

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

Rats and mice represent the lowest species of sentient animals suitable for this work. They are widely used for this type of work and so the results we produce will build upon a substantial body of existing information both from our laboratory and others. We wish to examine how the signalling properties of excitable cells in the brain change with age and how these changes impact on the ability of the brain to learn behaviours that are known to decline with age. We wish to compare these effects to those that take place in examples of unhealthy aging such as the neurodegenerative condition Alzheimers disease. We therefore need to use young adult animals and those that have aged naturally, as well as genetically modified animals that have been developed to mimic forms of neurodegenerative disease.

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

Two main types of experiments will be carried out. In the first, animals will be deeply anaesthetised using a gaseous anaesthetic and then killed rapidly to allow removal of brain tissue so that we can then use this tissue in experiments carried out in vitro. In a small number of experiments, recordings will be made from these anaesthetised animals to examine neuronal function in the complete brain. In the second key part of this project, we will take a different approach which is to record neuronal activity using optical techniques with a miniature head-mounted microscope. This will allow us to test the behaviour of these animals during the learning of tasks which require parts of the brain that are affected during normal aging and the onset of Alzheimers disease. For these experiments, mice will undergo one or more surgical procedures under anaesthesia that allow the injection of material that causes a genetically encoded sensor to be expressed in the brain. A small glass lens will be implanted

into the brain at the injection site and a miniature baseplate fixed to the skull to allow a small lightweight microscope to be temporarily clipped onto the animal. These are small enough for the animals to behave freely without the need for anaesthesia. The preparations for these recordings can be done with up to 4 short surgeries that may also involve the implantation of a miniature device for the supply of pharmacological agents designed to reverse the effects of aging or neurodegeneration. Individual surgeries take

approximately 1 hour to complete and animals are allowed 1 week to recover between surgeries with prophylactic pain control provided. Behavioural experiments take up to 30 minutes but may be repeated several times to establish whether animals learn. During these experiments, the microscope is placed onto the animal and it is held secure with magnets. Video streams of data are recorded during the animals' behaviour and analysed offline later. We are able to correlate the activity of neurones in the brain with behavioural performance and hence gather an understanding of how neuronal activity changes during aging and neurodegeneration and how this impacts on learning.

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

Some experiments are performed on animals that are deeply anaesthetised and they do not wake up after the experiment. Apart from the induction of anaesthesia, there are no expected adverse effects for these animals. For experiments where the purpose is to observe neuronal activity during learning, the mice need to undergo one or more surgical procedure. Animals are provided with prophylactic pain control during and after the procedure to minimise pain. Animals recover within hours of the procedures and generally start to behave normally very quickly. Animals that do not show normal behaviour are scrutinised by professionally trained staff including veterinary surgeons and if they cannot be made comfortable, they are humanely killed.

Part of our project involves the use of aged mice. Most mice age normally but as animals get older, they are more susceptible to conditions that are associated with old age. This might include the appearance of tumours or a general deterioration of the condition of the animal. Animals are monitored closely and parameters such as appearance and weight and checked. Humane endpoints are defined such that if an animal exceeds these defined stages, they are humanely killed if it is not possible to treat the situation.

We will also use mice that have been genetically altered to contain mutations that occur in humans that give rise to early onset forms of Alzheimers disease. These animals display cognitive decline at an age approximately 3 times earlier than normal mice. These mice will therefore be used to study the effects of Alzheimers disease but at an age where the neuropathological signs are present (> 4 months) but the behavioural events are evident but the harmful/adverse effects are minimal. These animals will be observed in much the same way as aged mice to ensure that they do not suffer 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)?

Surgery has some risk but from experience, approximately 5% of mice are likely not to recover from a single surgical procedure. Additional surgeries will add further risk but we will minimise this by limiting, where possible, the number of surgeries per mouse to a goal of no more than two. This is a feasible goal as our surgical and experimental techniques become more refined. Post-surgery, all animals are likely to experience some discomfort

but this will be ameliorated with analgesic treatment. Each individual surgery is likely to reach a mild severity but it is the need to carry out repeat surgeries that means that mice that undergo multiple surgeries are more likely to reach the maximum allowed severity level of moderate. This will be countered by careful peri-operative analgesia and a move towards reducing the number of surgeries to a minimum which we hope will be two. Nevertheless, it is likely that most of the animals undergoing two or more surgeries will reach the moderate severity limit which is the maximum for this procedure.

Mice will be aged up to 24 months but the majority (approx 75%) will be used for experiments at ages up to 18 months. At this time point, most mice do not display adverse effects or only experience mild effects. A small number of animals (< 25% of those animals that fall into the aged category) may be aged up to 24 months because the effects of ageing continue to increase with age and it may be necessary to examine this very old age group. Our group ranges for these ageing studies are <6 months, 12 months, 18 months and <24 months. Animals under 12 months are not considered aged and so would not expect to meet the maximum severity level for this protocol which is mild. By 18 months of age, the proportion of animals that might experience age-related symptoms will increase but from past experience, the numbers that are likely to experience the maximum severity rate of this protocol (moderate) are <10% of this particular age group. Where possible, we will use 18 month old mice as our maximum age group but as already indicated, age-dependent effects on cognition increase with age. Of the upper limit of 25% of all animals that might be aged up to 25%, we expect that 25% of these mice (also estimated from past studies) might experience symptoms that place them into the maximum severity rate for this protocol (moderate).

In studies using genetically altered mice that exhibit high levels of amyloid plaque deposition, we expect < 10% of the mice to display behaviours that approach the maximum severity level of this protocol (moderate) since the majority of mice will be used at an age where the pathology is present but the adverse effects remain minimal.

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

- Killed

Replacement

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

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

We wish to understand how the excitable cells within the brain communicate under normal circumstances and how their transmission properties change with learning. Using novel optical and electrical methods to assess activity in key areas of the brain associated with particular forms of learning, we will then examine how the properties of these cells change during the process of normal ageing and the onset of neurodegenerative conditions such as Alzheimer’s disease.

To achieve these goals, we need to be able to examine neuronal function in a realistic and preserved network in animals and tissues that have been aged and in clinically relevant models that display signs that are as similar as possible to those observed in Alzheimers disease in humans. Immortalised cell lines are not suitable for most of this work because they do not form realistic neural networks. Brain slices represent an important model for us because the networks are largely preserved and the slices afford access to single cell recordings, population recordings, easy drug access and modern imaging methods that provide more information than electrophysiology alone. However, it is not possible to link changes in behaviour to changes in single cells or networks of cells. For this, it is necessary to transition experiments into in vivo work where we can associate changes in cell and network properties with changes in behaviour. The use of genetically altered animals that display signs and symptoms of diseases, allied to in vivo work, provides us with the models necessary to understand and hopefully combat these diseases. Our approach to this is to use the most relevant model to achieve each aim whilst taking all steps possible to adhere to the principles of the 3Rs.

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

We have made extensive use of immortalised cell lines in the past and we continue to do so where possible. We also use mathematical modelling approaches to inform and thereby minimise the use of animals. However, there are not yet available models that can realistically model neuronal activity during behaviour.

Why were they not suitable?

Non-animal models are not yet sophisticate enough to replace the experiments detailed in this project. They are very useful for helping to predict the actions of drugs, for example, on relatively simple systems and this information informs further work using animals. However, models are limited by the information available and animal experimentation helps to provide that 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?

The number of mice used is higher than the number of experiments that we will conduct because it includes the process of breeding the mice. In some cases, the breeding approaches we use mean that mice are heterozygous such that a mouse containing the genetic alteration is bred with a wild-type animal. With this method, half of the animals will be positive and half wild-type. Some of the wild-type mice will be used as controls but the majority of the experiments included in this project will be using positive GA mice. We will also breed these animals with transgenic mice that are positive for a mutation of the

amyloid precursor protein. This will be a complex process involving multiple crosses to develop a stable line that expresses both SyG37 and APP knock in mutations.

Many of these mice will be used for the collection of brain tissue and the numbers over 5 years is based upon the numbers of experiments we know we can carry out per week to meet the objectives of the project.

A relatively small proportion of animals will undergo surgical procedures prior to behavioural testing and some transgenic animals will only undergo behavioural testing. Numbers are based upon the numbers required to ensure that statistical results can be relied upon.

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

We have set our objectives and experiments to work in a way that enables us to define the nature of experiments using methods that do not require either the use of animals of techniques that are not covered by this project licence. This first stage ensures that we only carry out work on animals that we are already sure there is a good scientific reason to indicate they will be successful. We have also, over the last few years, refined out experimental approaches to microscope development which means that our success rate is increasing so that we are more likely to achieve a successful outcome.

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

We regularly share tissue and several colleagues in our department use parts of the brain that we do not require. A single brain can yield at least 10 hippocampal slices, 5 cerebellar slices and 3 slices of brain stem, for example. We have developed a computer model based on our work looking at presynaptic calcium to test in silico the effects of drugs that alter, for example, ion channel properties. These results can provide an insight as to which drugs might be useful in our tests, We can then try these drugs in vitro and if successful, use them in vivo. This approach allows us to eliminate unnecessary experiments early on in the scientific process and only perform experiments on awake animals that are very likely to yield useful 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.

Over the last few years, we have developed a transgenic mouse that expresses a calcium sensor selectively at presynaptic terminals in the brain. We have used this mouse model to examine the nature of synaptic transmission in the hippocampus and cerebellum and most recently to show that during the ageing process, calcium levels in the presynaptic terminals becomes disrupted leading to changes in the release properties of synapses and consequently changes in transmission strength and in the ability of synapses to change their strength. Concurrently, we have shown that these aged mice have a reduced ability to learn in behavioural tasks associated particularly with spatial navigation that involves and requires hippocampal integrity. By expressing this sensor in mice, it is possible to prepare brain slices from these animals and record, using optical techniques, directly from the tissue without the need for complex electrophysiology or injection of dyes or other agents to measure neuronal activity. This greatly increases the success rate of experiments and allows more experiments to be performed from a single animal, thereby addressing the 3Rs directly. The use of in vitro tissue allows animals to be used with minimal suffering.

These mice also pave the way for more sophisticated in vivo experiments. It is possible to measure calcium signals in these behaving animals using miniaturised microscopes to examine the contributions of presynaptic calcium to neuronal activity during behaviour. These can be combined with the application of other sensors, such as GCaMPs which are designed to be expressed in postsynaptic structures so that pre- and post-synaptic activity can be compared.

We have developed a miniature microscope that weighs less than 1.5 grammes and clips onto a small baseplate fixed to the skull of mice. The mice appear to behave normally after a short period of acclimatisation. This method allows us to record from a field of neurones in real time during behaviours that require learning. Compared to some electrical recording methods, this optical approach provides information from tens of cells simultaneously so we can understand how networks of cells interact rather than just one cell at a time. The animals become rapidly acclimatised to the experimenter. If the animals are distressed, they will not perform.

Ageing is a natural phenomenon. If animals start to show adverse effects, then they are treated and if that is not successful, then they are humanely killed so they do not suffer. Whilst animals age, they are well cared for by the staff at the facility, observed regularly and provided with food and water and in cage 'enrichment.

To understand how the brain changes during unhealthy ageing as, for example, during the development of Alzheimer's disease, we will use a transgenic mouse model that over-expresses amyloid leading to amyloid plaque formation. By combining this mouse strain with our own SyG37 presynaptic calcium model, we will be able to examine the contributions that presynaptic calcium make to changes associated with the development of this neurodegenerative disease. This new model will assimilate the advantages of our mouse model in recording presynaptic calcium with a disease model that mimics the pathophysiology of Alzheimer's disease and combined will advance our understanding of the process of 'unhealthy' aging.

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

Rats and mice are mammals. Their brain structure is similar to that of humans and they undergo and perform behaviours that are reminiscent of those that humans carry out. It is possible to use less sentient species for some experiments involving behaviour but the availability of transgenic mouse models and the similarity of their brains to humans make them a realistic model for aging and agerelated diseases.

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

Animals receive very careful and thorough post-operative care. They are observed immediately after surgery and treated immediately if they do not show appropriate signs of recovery or if they show signs of pain or distress. Peri-and post-operative analgesia is provided. Our miniscopes have been adapted recently to reduce the size of the implanted lens to minimise the damage to the brain caused during implantation and to increase the success rates of experiments.

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

Prior to experimentation, we will conduct a systematic review of the literature to ensure that the experimentation is necessary and then to ensure that the approach we use is likely to yield the expected results.

In experiments involving surgery, we will use best practice in all aspects of the work including aseptic surgical techniques and the use of peri-operative analgesia to minimise pain and suffering. We will, where possible, use implantable minipumps to standardise chronic drug administration. Although these pumps require a surgical procedure for implantation, their use prevents the need for daily injections and provides a constant, controllable level of drug administration.

We are constantly refining the design of our miniature microscopes to reduce the size, weight and to improve their ease of use for the experimenter. With practice, they can be clipped into place easily as they are held in place with magnets producing a stable recording that lasts for many weeks/months. By adapting the miniature lenses that we use, we can now avoid having to remove large areas of tissue above our recording site and thereby improve the repeatability of our experiments and reduce the influence of the experimental method themselves on the experimental outcome.

The use of transgenic mouse strains allows us to use the most realistic models of human diseases available and by combining these models of neurodegeneration with our SyG37 strain, we can assess neuronal presynaptic function easily without the need for more complex methodologies.

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

We undergo regular professional development which includes ensuring that we remain aware of the principles of the 3Rs. We will discuss our work with the named persons, keep abreast of developments through NC3Rs and take advantage of advances in technology that will reduce the numbers of animals used and enhance the success rate and amount of data obtained.

11. Cryopreservation, Breeding and maintenance of genetically altered mice as a service

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Cryopreservation, Re-derivation, Breeding, Rodents, Health status

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Maintain a high-health status of rodent colonies as required by many ongoing scientific projects, using the practice of embryo re-derivation.

Preserve and archive important genetically altered rodent lines by cryopreservation to ensure prevention against loss as well as to avoid unnecessary 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 acc**rue after the project has finished.**

Why is it important to undertake this work?

Animal models remain indispensable tools for studying disease biology and physiological processes. One of the reasons the mouse remains a valuable model for such study is the ability to manipulate its genome. Therefore, transgenic and gene targeted mice will continue to be required.

Where possible, we would import genetically altered (GA) lines into our facility from sources that provide high health status animals devoid of pathogens. However, this is not always possible as many academic Institutions both national and internationally do not maintain high health status animals compared with those housed in our facilities. Such animals before entering our main experimental facility are cleaned by embryo re-derivation. Once the health status of imported animals becomes comparable with existing animals, breeding and experimental work can start. In addition, rather than import live animals from other sources that cause travel stress to animals, we will have the ability to import frozen genetic material (sperm or embryos) and produce a line of interest by using non- conventional breeding practices, such as surgical embryo transfer in suitable female mice.

Value of cryopreservation of sperm and embryos. Generation of a bank of frozen embryos and/or sperm from all modified lines would reduce breeding of mice and ensure a repository is available for future use. Cryopreservation of GA animal lines contribute significantly towards implementing 3Rs, but especially Reduction.

The present project license that provides the above services to the scientific community is held by a senior academic and will expire in January 2021. Specifically, these services include provision of breeding high health status genetically altered (GA) mice by conventional breeding or by embryo transfer and cryopreservation of genetic material from the lines that need to archived or are still needed by the research community.

Several research groups who are familiar with my work in the field have indicated that Animal Facility management should continue to provide these services through a dedicated project licence for a further 5 years.

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

Maintain a high-health status of rodent colonies as required by many ongoing scientific projects by the practice of embryo re-derivation

Preserve and archive important genetically altered rodent lines by cryopreservation to ensure prevention against loss as well as to avoid unnecessary breeding

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

We can provide the skills, knowledge and equipment required to efficiently produce transgenic mice with the minimum of animal wastage.

Cryopreservation service. Archiving frozen embryos and/or sperm from all GA lines would reduce breeding, and therefore the number of excess animals being produced. It will also ensure a repository is available for future use to guard against the potential loss of a line which would be difficult to replace.

Maintaining a high animal-health status. Required for many scientific projects by preventing any import of pathogens into the animal facility by the practice of embryo re-derivation.

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

The staff members providing the Transgenic Service are familiar with the mouse lines we commonly use and are highly experienced in the techniques involved in transgenic mice production and the breeding of GA mice. The skills and methods are continually being improved and optimized. No individual lab would be able to devote an equivalent amount of time to increasing efficiency and troubleshooting.

Species and numbers of animals expected to be used

- Mice: No more than 14,300 rodents will be used over the 5 year project time frame. This is broken down into 13,000 (max) undergoing mild severity and 1,300 (max) undergoing moderate severity procedures.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 production of GA mice is technically difficult. We can provide the skills, knowledge and equipment required to efficiently produce transgenic mice with the minimum of animal wastage. 1.

Cryopreservation service. Archiving frozen embryos and/or sperm from all modified lines would reduce breeding, and therefore the number of excess animals being produced. It will also ensure a repository is available for future use to guard against the potential loss of a line which would be difficult to replace. 2. Breeding and maintenance of GA lines. 3. Maintaining a high animal-health status.

Required for many scientific projects by preventing any import of pathogens into the animal facility by the practice of embryo re-derivation.

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

- Vasectomy
- Under general anaesthetic the vas deferens will be exposed via the scrotal approach. Each duct will be severed, resected, sutured and/or cauterised, the wound closed, and the animal allowed to recover
- Embryo recipients
- Surgical or non-surgical Implantation of genetically modified embryos into the oviduct or uterus of pseudo pregnant female, the animal allowed to recover, give birth, the litter is kept but the female is killed by a Schedule 1 method
- Superovulation
- Administration of substances by intraperitoneal injection (e.g. gonadotrophin followed about 48 hours later by luteinizing hormone. Maximum volumes 0.5 ml of each) All mice are killed by a Schedule 1 method within 7 days of the initial injection and oocytes/embryos are harvested post-mortem

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

All surgical procedures will be carried out using sterile techniques to minimise the potential risks of infection to the animal. All animals will be given pain relief before any surgery to avoid pain or potential discomfort. Animals will be either transferred onto other PPL’s to be used in further projects, or humanely killed once they reach their set end point.

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

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

The majority of the work will be under a mild severity limit with very little likelihood of any adverse effects to the animals. Any procedures that are carried out under moderate rated protocols have very little chance of adverse effects.

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 some scientific projects it is not possible to replace the use of rodents. When investigating disease and developmental processes, the complexity of a whole organism cannot always be recapitulated using alternative in vitro systems. The tissue, cell and molecular interactions involved in such complex processes cannot be examined in their entirety in vitro.

The genetically altered mice generated and bred under this PPL will be investigated for phenotypes and processes that cannot be examined in any other way. All animals bred on this PPL are destined for use in another PPL and the case for that particular model will have been made, and approved, separately in that PPL and by that institute's Animal Welfare and Ethical Review Body.

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

It is not possible to replace the use of rodents

Why were they not suitable?

As we need to freeze sperm or embryo to archive lines, this is only possible using live rodents.

Reduction

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

numbers consistent with scientific objectives, if any. These 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 estimates from numbers used under the previous licence and in discussion with users of the facility, data from cryopreservation and embryo transfers that is stored on our database.

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

Looking at breeding data provided by our database, HO returns and data from cryopreservation and embryo transfers that is stored on our database.

The breeding data is used to work out breeding calculation which uses litter size, litter interval and how much stock that needs to be produced, which will give an idea of how many breeders need to be set up.

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 proposed in this programme of work are based on reasonable estimates of generating the required GA mouse lines for the facility over the next 5 years. The advantage of using a centralised transgenic service is that it will decrease the overall number of mice used to generate GA lines. The reasons for this are:

- The availability of highly skilled workers will ensure the lowest number of animals possible will be used.
- Central coordination of animal stock production allows the most efficient use of breeding stock. Excess mice or embryos generated for one project can be used in other transgenic projects.
- Sharing of sterile male mice between projects requiring generation of pseudo pregnant females.
- Making use of our expertise in the areas of sperm and embryo freezing to archive all mutant lines, such that a stock of live mice for each line are not required to ensure lines are not lost.
- The strains that are being used in the facility have been chosen because they are the most efficient for production of transgenic mice.
- New techniques are continually tested and adopted to reduce animal usage significantly.

Refinement

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

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

The majority of the work will be under a mild severity limit with very little likelihood of any adverse effects to the animals. Any procedures that are carried out under moderate rated protocols have very little chance of adverse effects. However, all surgical procedures will be carried out using sterile techniques to minimise the potential risks of infection to the animal.

All animals will be given pain relief immediately after any surgery to avoid pain or potential discomfort. Animals will be either transferred onto other PPL's to be used in further projects, or humanely killed once they reach their set end point.

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

Most of the rodents being used have to be at breeding age (6-8 weeks) to carry out this work, with the exception of the superovulated females which are 4 weeks+.

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

The availability of highly skilled workers will ensure the lowest number of animals possible will be used.

Equipment is being used that will maximise the efficiency of transgenic production. Such equipment is costly and could only be purchased by a central service unit.

Central coordination of animal stock production allows the most efficient use of breeding stock. Excess mice or embryos generated for one project can be used in other transgenic projects. Sharing of sterile male mice between projects requiring generation of pseudo pregnant females.

Making use of our expertise in the areas of sperm and embryo freezing to archive all mutant lines, such that a stock of live mice for each line are not required to ensure lines are not lost. The strains that are being used in the facility have been chosen because they are the most efficient for production of transgenic mice.

New techniques are continually tested and adopted to reduce animal usage significantly. we are hoping to do further training on the Subcuticular Stitch and Wound Glue method shown on training course, which would replace the need for autoclips on the embryo transfer females and further training on nonsurgical transfers at the moment it would need to be a mixture of both surgical and non-surgical methods so we could compare the data.

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

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assessment of transgenic rodents (mice). Journal of Applied Animal Welfare Science 3:127.

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Behringer R. et al., (2014) Manipulating the Mouse Embryo: A Laboratory Manual, 4th ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

Jackson, I.J. and Abbott, C.M. (2000) Mouse genetics and transgenics: A practical approach. Oxford University Press.

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

The NC3R's information is sent out in a monthly newsletter, looking out for training sessions which would help implement and improve my work and practices.

12. Using zebrafish to understand neurodegenerative disease and develop 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

zebrafish, development, disability, therapy, mechanism

Animal types	Life stages
Zebra fish	embryo, 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 increase our understanding of diseases and the functions of the mutated proteins that cause those diseases, as well as providing disease models for therapeutic discovery and 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?

The project focuses on rare inherited diseases, which together affect about one in every 10 people, even though each disease has an incidence of less than 1 in 2000 people. They frequently cause premature death in childhood and the vast majority have no approved treatments. We will also study diseases that have a more complex origin, in which the same genetic factors interact with environmental factors. There is a huge need to find treatments for both inherited and complex diseases.

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

We expect to have increased our understanding of the function of three or more proteins involved in childhood neurodegeneration and epilepsy, including understanding how those proteins interact with other processes within the cell and how disease arises. We plan to generate several disease models, and by studying them we will better understand the disease process. One or more chemicals will be tested to see if they treat any of the childhood neurodegenerative diseases that we are studying. We will publish our findings.

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

In the short-term scientists will benefit from increased understanding of childhood neurodegenerative diseases including the normal function of the protein that is mutated in the disease, and the effect of the disease on processes occurring within cells and a whole organism. This will lead to better treatments for patients in the long term.

The availability of additional disease models will enable treatments to be tested on more models so that the research community can be more confident that the treatment will work on patients, who will benefit in the medium term.

The availability of zebrafish disease models that can be used to test thousands of chemicals in a relatively short amount of time means they can be exploited to find novel chemicals with potential to treat the disease. This will benefit scientists in the short-term and patients in the long-term.

We found a chemical that appears to treat seizures in CLN2 disease. Knowing how this chemical has its positive effect will support efforts to bring the chemical to the clinic as a drug in the medium term, and provide researchers with new knowledge in the short-term.

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

We will publish our work as open access. We plan to publish both positive and negative results. We will present our research at international conferences.

We collaborate with others to increase the impact of our research.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 21400

Predicted harms

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

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

Data from zebrafish can be extrapolated to other vertebrates due to a high level of similarity. This means that it is valid to use the zebrafish to model disease, investigate what is going wrong in the disease and search for and test novel treatments. The rapid, external development of zebrafish enables the vast majority of experiments to be performed on developing zebrafish that do not feel harm, and without harming the mother. Of the available vertebrate models, zebrafish have the lowest brain function.

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

We will study existing and new disease models, which we will create. To create new models, we inject the one cell embryo with agents to specifically alter a targeted gene. We select healthy embryos and grow these to adulthood. We then select adult fish carrying this genetic alteration in their sperm and eggs, and breed from those fish to generate subsequent generations.

During these breeding programs, we need to be able to identify fish carrying the genetic alteration from wildtype fish, so we take a small number of cells (either from their fin which regenerates, or from their mucus on their skin) and analyse the gene for the alteration. Once we have identified the genetically altered fish, we incross them to make fish that have two genetically-altered copies of the gene, which we expect to show signs of the disease at some point during their life. We then assess those potential disease models to see if they do actually show signs of the disease, and if so, we study them further, and set a humane endpoint.

To understand what is going wrong in the disease, we sometimes need to manipulate other processes as well, either using chemicals or by altering other genes. We then examine the fish to see if this has made a difference. We can examine their movement or behaviour, or an invisible process happening within, which we visualise with a specific label, most often on fish that have been sacrificed for their tissue.

To search for treatments, we use disease models at the embryo stage to screen large numbers of chemicals, and check to see if the signs of the disease are reduced by adding the chemical to the water the fish are in. If we already have good reason to believe that a chemical might make a good treatment, we might use adult disease models but we will use embryonic and larval models first if possible. In this case we might bathe the fish in the chemical, or inject it. If our results are similar to those of our collaborators using other

species or patient cells, we can be more confident that the treatment will be beneficial for patients.

As a practical way to reduce the numbers of fish kept alive, we take sperm from adult males and freeze it. We can then use this to re-derive the strain several years later using in vitro fertilisation (where eggs are taken from a female adult and mixed with the sperm to generate embryos that will grow into adult fish).

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

Most adverse effects are expected to be transitory eg when taking mucus or fin samples there may be slight discomfort, which we treat with pain killers. Sometimes we will need to anaesthetise the animals eg when taking mucus or fin samples, and there is a small chance of an adverse reaction, in which case we will euthanise the fish straight away. It is possible that some experiments could result in infection, in which case we will treat with antibiotics or euthanise the fish. Massaging the belly to release eggs or sperm could also result in damage to internal organs if not gentle enough, in which case we will euthanise the fish as soon as this is noticed.

The disease models are likely to show one or more of the following signs: obviously small eyes; obvious weight loss; discolouration; abnormal swimming, behaviour, posture or balance; abnormal gill movement; unusual interactions such as aggression. We will euthanise all fish which have shown these symptoms for 4 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)?

About 50% of fish will experience mild severity and 50% 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?

To model a disease, investigate what is going wrong, search for therapies and test those therapies, you need all the relevant tissues present. The diseases we work on predominantly affect the nervous system but there is increasing evidence that they affect the heart, so we expect problems to arise in other tissues if we manage to treat the brain and improve survival. Furthermore, in some of these diseases, it is known that patients

generate antibodies in their blood that bind proteins in their brain. Hence, a full understanding or the disease and treatments will only come from studying intact animals.

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

We employ non-animal alternatives and non-sentient animals where possible. These include performing experiments in cell models (traditionally used mammalian cells, patient cells, human stem cells, the social amoeba), invertebrates such as the fruit fly and the nematode worm, frog oocytes and/or embryonic zebrafish depending on their suitability for the experiment. We also take tissue from euthanised zebrafish and grow the cells in a dish.

Why were they not suitable?

These methods do not replicate the complex environment seen in a vertebrate but they do give us some information so we do these experiments to inform what experiments should be performed in zebrafish.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 expect to use up to 8000 of these fish for breeding purposes to supply embryos for experiments. This is based on 40 different strains (some are combinations of several strains) with no harms caused by the genetic manipulation. Zebrafish are healthier when in groups of 20 or more, and new generations are needed about every 6 months - the old generations are not euthanised until the new generations are genotyped (usually as adults). For some complex strains, only a small proportion of the fish are the correct ones but this is not known until adulthood when we can check their genes. Over a 5 year period, this adds up to 8000 fish.

We expect to use 8000 (over a 5 year period) genetically-altered strains for simple experiments where we document the harmful effects of the genetic alteration. Each experiment requires a pilot study to calculate the number of animals required for a full study, and then each study should be replicated three times to ensure reproducibility. Assuming 75 animals (25 animals in each of three groups) per replicate, this allows for about 35 experiments over 5 years.

We expect to use 4000 (over a 5 year period) genetically-altered strains for complex experiments where we document the potentially harmful effects of the genetic alteration in combination with a drug or chemical treatment, for example. Each experiment requires a pilot study to calculate the number of animals required for a full study, and then each study should be replicated three times to ensure reproducibility. Assuming 150 animals (25

animals in each of six groups) per replicate, this allows for about 9 experiments over 5 years.

We will need to generate new strains of zebrafish and estimate 1000 animals will be needed. This is assuming 5% of animals will be able to transmit the new genetic alteration to their progeny and that we may make up to 50 strains.

In order to preserve newly created strains, we expect to use 400 animals, which is approximately 10 animals for 40 new strains or combinations of strains.

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 Experimental Design Assistant to design experiments using zebrafish embryos and will continue to use it for all zebrafish experiments. This has led to us reducing bias. For each experiment we also write 1) the objective, 2) a detailed description of the method and reagents needed (and ensure they are available) to minimise variance between researchers, 3) a calculation of the numbers of animals needed based on small (pilot) studies, and 4) a description of the analysis method and the statistical test to be used.

We preserve sperm to reduce live animals, negating the need to recreate strains or combinations of strains, and reduce genetic drift. We use live animals for egg and sperm production to reduce numbers of animals euthanized for this.

We aim to try new, less harmful methods at earlier stages in zebrafish development for detecting the genetic alterations. Fish are not harmed and can be raised to adulthood without having any harm. Significantly, only fish of the required genetic alteration need to be raised to adulthood, reducing numbers.

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

Pilot experiments will be used to generate data to determine a suitable number of animals for a full experiment. For experiments using embryos, we usually find that 10 per group is acceptable. Simple experiments comparing wildtype to a disease model tend to require less animals than an experiment where there is also a drug or chemical treatment, so in these cases, more animals are usually needed. For experiments new to us, we discuss animal numbers and data analysis with statisticians in advance.

We estimate the number of animals needed and breed what is required plus 20% to account for deaths during larval stages. Once zebrafish are checked for their genetic alterations, those not required for experiments or breeding are euthanised. We aim to determine the genetic alterations using the least harmful method possible.

We offer to share spare animals and tissue at our institution.

We do not keep animals beyond 18 months old and frequently euthanise them when we can.

Refinement

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

Which animal models and methods 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 as there is now a great deal of evidence that data from this vertebrate is relevant to humans. This is true for models we have generated to date for CLN3 disease, CLN2 disease and EAST syndrome are no exception. We also have evidence that a novel drug first tested in cells works similarly in mouse and zebrafish to reduce CLN3 disease symptoms. The fast, external development of zebrafish means that many disease models can have clinical signs during embryo and larval stages, and these can be viewed easily without harming the mother. Embryonic and larval zebrafish are not sentient and do not feel harm so are not classified as animals when used for experiments and therefore their use is not regulated.

The CLN2 disease model will be used at the unregulated age. The CLN3 disease mutant model causes only mild symptoms (thinner retina and yellow colour) in adults. We do not yet know when the new disease models will display symptoms, but we aim to make models that replicate the genetic change in humans as best we can so that the model is as relevant to the disease as it can be.

The majority of animals will not experience more than mild issues due to experiments, minimising suffering. Some animals will experience moderate harm, because they are used for experiments that require us to observe the harmful consequences of the genetic alteration, though we aim to limit the harm caused as much as possible by choosing to study the earliest symptoms. Occasionally, we only have one animal for a particular strain. To avoid single housing, we will house them with animals of another genotype that have a visible difference such as fin length, pattern or coloration.

Some of the animals that will experience harm from the genetic manipulation will be further manipulated with genetic material, chemicals or drugs, or by transplantation of tissue from one animal to another. Such experiments are required to show a drug treats the disease, or that a disease process really has negative consequences and should be targeted in treatments, or which cells need to be corrected by gene therapy. Transplantations will be performed during unregulated stages to minimise harm and because the tissue integrates better into the host at this stage. Drug, chemical or gene therapy treatments will be first tested on unregulated zebrafish. The least invasive methods of delivery will be used where possible (bathing, in the food, through the mouth, then injection). In these experiments we also aim to limit harm by choosing to measure the earliest harmful effects. We will create

new disease models and genetically-altered strains. Only healthy fish are raised to minimise harm.

Fish will be examined for the effects of their genetic alteration or treatment. The majority of these investigations will be observations of normal behaviour and locomotion or tests on the tissue after euthanasia. We will perform some brain function tests that require anaesthesia or restraint but are otherwise not harmful. The dose of anaesthesia is carefully checked for the first fish (ensuring a quick and healthy recovery after the procedure) and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish.

The fish in all the genetically-altered strains will need their genetic alteration identified. We will use the least harmful method and the earliest stage possible. When tissue samples are required, we will preferentially use skin swabs over fin clippings. Both of these methods use anaesthesia with recovery, but skin swabs are thought to be less harmful. We have recently begun using skin swabs and will need to show that the results using this method is as good as with fin clips for each strain before we can use skin swabs exclusively.

The dose of anaesthesia is carefully checked for the first fish (ensuring a quick and healthy recovery) and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish. Pain killer is used prior to, during and after fin clipping.

We will need to preserve sperm, which allows us to freeze these strains and maintain less fish alive. These strains can later be resurrected using in vitro fertilisation. Again, the dose of anaesthesia is carefully checked when taking sperm or eggs. Fish recover and can be used to provide sperm or eggs once more, meaning less fish are required. Currently, experts at another university preserve sperm for us. We aim for them to train us in this method.

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

Where possible, we will use animals at more immature life stages which are less sentient. This will depend on the stage when the genetic alteration causes symptoms or clincal signs that are measurably different from wild type zebrafish. When we do not need to measure a clinical sign, we will measure changes that are observed in tissue from animals euthanised prior to the age when the clinical sign is evident. When we do need to measure a clinical sign, we will design experiments to assess as many changes as possible (both live and under terminal anaesthesia) to gain maximum data from each animal.

We favour the use of zebrafish over invertebrates as the zebrafish brain is considerably more similar to the mammalian brain. As zebrafish are the genetic vertebrate model with lowest sentience, they are also our preferred vertebrate for experiments at regulated stages. We feel that the zebrafish is the most refined model for the research questions we are addressing.

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

When studying adverse effects, we aim to limit the harm caused as much as possible by choosing to study the earliest relevant consequences.

Occasionally, we only have one animal for a particular strain. To avoid single housing, we will house them with an animals that have a visible difference such as fin length, pattern or coloration.

Transplantations will be performed during unregulated stages to minimise harm and because the tissue integrates better into the host at this stage.

Drug, chemical or gene therapy treatments will be first tested on unregulated zebrafish. The least invasive methods of delivery will be used where possible (bathing, in the food, through the mouth, then injection.

We will create new disease models and genetically-altered strains. In this method, only healthy fish are raised, to minimise harm.

Once we have created a new genetically-altered strain, we will develop a score sheet for that strain so expected clinical signs and symptoms are known and specifically looked for.

The dose of anaesthesia is carefully checked for the first fish and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish.

To detect the genetic alteration, we will use the least harmful method and the earliest stage possible. When tissue samples are required, we will preferentially use skin swabs over fin clippings. Both of these methods use anaesthesia with recovery, but skin swabs are thought to be less harmful.

We have recently begun using skin swabs and will need to show that the results using this method is as good as with fin clips for each strain before we can use skin swabs exclusively. We use pain killers when we take fin samples.

We aim to try new, less harmful methods at earlier stages in zebrafish development for detecting the genetic alterations. Fish are not harmed and can be raised to adulthood without having any harm. Significantly, only fish of the required genetic alteration need to be raised to adulthood, reducing numbers.

The protocols state the minimum amount of monitoring of zebrafish that should be provided. Monitoring will be increased if any fish respond differently than normal to the procedure.

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

We will follow Home Office guidance. For published methods, to ensure best practice and least harm, we will discuss new methods with the Named Animal Care and Welfare Officer and possibly the Named Veterinary Surgeon.

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

We seek advice from large zebrafish facilities and keep abreast of latest refinements via the British Association of Animal Husbandry, the European Zebrafish Meeting, the Lab Animal Science Association, the NC3Rs, the Institute for Animal Technology and the journal Zebrafish. We receive newsletters and bulletins from these groups. We search the literature for more refined methods. I am a committee member for EUFishBiomed, members of which have recently reviewed use of anaesthetic.

We have meetings every few months with other research groups using fish and the staff that care for the fish and present our research so they can feed back on any best practice they are aware of.

We will keep up to date with changes in the ARRIVE guidelines for the reporting of animal experimentation.

When we find a new method we believe to be best practice, we contact the developer and arrange for training.

13. Targeting brain tumour metabolism to improve treatment outcomes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Intracranial, Brain Tumours, Therapy, Metabolism, Xenograft

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 the efficacy of new treatment strategies, which have been fully tested in vitro, in animal models of brain tumours. Data generated will provide robust pre-clinical information to inform the development of clinical trials for brain tumour 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?

Brain tumours will kill around 11,000 people every year, many of them under the age of 40, and is the second leading cause of cancer deaths in children and young adults. Current standard of care is only palliative and includes surgery, radiotherapy and chemotherapy; however, these tumours typically recur within a year and are resistant to further treatment. Furthermore, the limitations posed by the blood brain barrier prevents the use of many forms of chemotherapy that have shown success in other cancers. In

addition, the high degree of variability between brain tumours from different patients makes it difficult to identify effective therapies. There is therefore an urgent need to identify new treatments that are more effective than current standard of care, or enhance the standard of care. Treatments targeting tumour metabolism, an alteration shared by virtually all cancers, could provide better outcomes for these patients.

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

Our work will generate fully characterized mouse brain tumour models using samples from patient tumours. These models can be utilized to test new therapeutic modalities by our group, and nationally and internationally by other brain tumour researchers. We will gain valuable information on the electrical and chemical signals that occur between the tumour and its environment and how these signals change upon therapeutic interventions. The project will also lead to high impact publications and inform the development of new clinical trials for brain tumour patients. We also have the potential to patent some of the therapeutic ideas generated.

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

Patients with brain tumours will be the primary beneficiaries of this project, as we are specifically testing new treatments on animals with brain tumours. However, we hope that any new treatments we develop will also be of benefit to patients with other types of cancer, particularly since the tumour cells used will have characterized genetic alterations that can occur in other forms of cancer

Moreover, brain tumour scientist around the world will also benefit by having access to mouse models that will be generated

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

We will collaborate with other groups, seeking advice and expertise where necessary. We will also collaborate with other scientists to test new drugs or derivatives of drugs in animals, as well as pharmaceutical companies that have new drugs to test. Our knowledge will routinely be distributed to all research and clinical personnel via monthly research meetings, as well as to other members of our faculty interested in cancer though inter-department talks. We will present our findings at national and international conferences to further disseminate our knowledge across the world. We will generate high impact publications allowing dissemination of our findings to other interested scientists and clinicians who could potentially expand the use of these therapeutics into additional clinical trials.

In addition to disseminating our results to the research and clinical community, we regularly disseminate news of our progress to public and private donors.

Species and numbers of animals expected to be used

- Mice: 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.

To reliably assess the success of new treatments for brain tumours, we need to have the full complexity of a living, breathing animal. In particular, we need to make sure that any new treatment is safe, that it can get in to the brain, and that it has efficacy on brain tumours. This is easily achievable using mice.

With their relatively short lifespan, we can quickly raise animals to adulthood, and implant a brain tumour to treat. Due to their small stature, adult mice are easy to perform surgical procedures on. We will be using adult mice because our research focus is adults with brain tumours. By using bioluminescent imaging, we can follow the fate of the tumour in terms of its growth and regression and avoid unnecessary prolonged harm to the animals from the tumours becoming too big. Furthermore, scientists have employed mice for brain tumour research for years and so methods used are already very well established.

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

To test any new treatments, a mouse must first have a tumour implanted. This will normally be carried out by an injection of tumour cells directly in to the brain. Mice will be anaesthetised so that they do not feel any pain or discomfort during surgery. A small incision is performed in the scalp, and a hole is drilled in the skull. A small glass needle is then carefully inserted, and cancer cells are injected directly in to the brain. The skin is then sutured to close the wound, and the mice are taken off anaesthesia and allowed to recover in a warm chamber. When they have woken up and are walking about as normal, they will then be returned to their normal housing. The whole procedure normally takes about 1 hour. The mice will be given pain relief, and both the skull and the skin will heal on their own within 2 weeks, when the stitches can be removed.

After 5-7 days, the mice will be anaesthetised again and imaging will be performed on them. The need for reducing the post-op recovery time to 5 days in some instances will be necessary due to the fact that different tumour cell lines have varying growth rates. Therefore, imaging at this earlier time point will help to avoid the tumours from becoming too large before treatment in initiated. However, any reduction in the 7 day post op recovery period prior to further regulated procedures will only be carried out with the written approval of the NVS and clear justification will be provided for this need. All imaging methods are non-invasive. The mice may receive an injection of a chemical to help us image them. The imaging will typically last 30 minutes, after which the animals will be returned to their normal housing.

Imaging will take place once or twice per week over the entire course of the experiment. The frequency of administration of anaesthetic for imaging is not expected to have a significant impact on the metabolism of the tumour as the duration of anaesthesia is short, lasting no more than a maximum of 30 minutes from induction to full consciousness. In our previous work, administration of repeated anaesthetic had no effect on the treatments tested.

After we have confirmed the presence of a tumour, the mice may be given one of a number of different treatments. The mice may be anaesthetised again and treated using radiation. This is also non- invasive; the animals are shielded to protect their body, leaving only their heads exposed to radiation. This will be done once per week for between 2 and 8 weeks. Animals may also be given an injection of one or two drugs to treat the brain tumours. This will generally not require anaesthesia, and may be given once per day for up to 4 weeks.

We will continue to monitor the animals and image the tumours so that we can see how quickly they are growing. The length of time animals will be kept alive will be dependent on tumour size. Mice receiving no or minimal treatment tend to have large tumours, and they will be humanely killed before they experience major symptoms. Mice that are successfully treated may be kept alive for as long as they remain healthy and the tumour size remains small. However treated animals may also be killed at early time points to follow changes in the tumour composition as a result of the treatments.

Some animals with brain tumours will undergo surgery under terminal anaesthesia to expose the brain. A probe will be inserted in the brain such that it spans both the tumour and surrounding healthy tissue. This will enable us to study changes in electrical and chemical signals between the healthy brain and tumour.

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

The injection of cancer cells into the brains of mice is an invasive surgical procedure. However, the animals will be anaesthetised and will not feel any pain or discomfort. To manage the pain caused by the wound that is created, animals will be given pain relief. The pain is not expected to last longer than 24 hours. Animals may experience some weight loss after surgery, but from previous experiments, this is only transient and animals regain the weight within a week. The development of the brain tumour itself will cause some distress but since this is a necessity to test new treatments, mice will be closely monitored to make sure they are not suffering any obvious physical signs of discomfort or symptoms directly related to the tumours e.g. difficulty walking. These may be present for up to 24 hours, but such symptoms will not be allowed to persist beyond this time as animals will be killed.

Animals undergoing surgery to expose the brain and the insertion of probes for brain monitoring will be terminally anaesthetized so will feel no pain 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)?

As most of the animals will receive an injection of cancer cells, followed by either imaging, treatment or both, they will all fall in to the "moderate" severity category. We expect most of the animals to experience moderate severity due to the surgical procedures themselves,

as well as due to the presence of tumours. However, we have all of the appropriate refinements in place to make sure any pain or discomfort associated with these procedures is minimised.

The severity of the surgery to expose the brain for electrophysiological monitoring is moderate however it is performed under terminal anaesthesia and animals will feel no pain or 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?

Whilst we can test many different treatments in cells grown in the lab using different techniques, we are not yet able to fully replicate the complexity of the human body. New treatments need to be tested on animals to make sure that they are safe, and that they work properly. Only once we have completed these pre-clinical studies can treatments be put forward for testing in humans.

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

We have used cells grown in the lab to test our new therapies. This includes using normal, healthy human cells to test if a drug is toxic, and using cancer cells to see if a drug stops them from growing or kills them. We have also considered using computers that simulate cells and animals, to test if treatments are likely to work.

Why were they not suitable?

A tumour is not made up exclusively of cancer cells, they comprise many different types of cells, some of which help the tumour grow by providing nutrients, some hiding it from the immune system, and some laying dormant, ready to attack the tumour if activated. In addition, tumours in the brain sit behind a barrier called the blood brain barrier, which filters out lots of chemicals that aren't allowed to pass in to the brain, including many types of chemotherapy. It is these complex relationships that cannot be fully replicated in the lab. We require the complexity of an animal to fully replicate these conditions, so that we can properly test new treatments and prove that they work. In addition, computers are not yet smart enough to be able to simulate every possible reaction to a treatment that a cell or animal could have.

Therefore, these experiments must be done in animals to be able to get a full picture, so that we can safely inform clinical trials in humans.

Reduction

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

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

The numbers estimated here come from a well planned programme of work that aims to test new identified treatment regimes. The project has multiple objectives which we aim to achieve over a 5 year period. On average 150 mice will be used per year. Each experiment will require approximately 20 mice in order to compare new treatments to those already used in the clinic. This is necessary to demonstrate that our treatments are superior than those currently available. Taking this into account, 150 mice per year only equates to 7 experiments, i.e. less than one per month. We believe this number will be sufficient to develop new models of brain tumours and test new treatments as we develop them, with the ultimate aim of progressing these into human clinical trials as soon as possible.

Although we have estimated that we would need a total of 750 mice over the course of this project licence, we have requested an excess for the following reasons:

as a contingency for failure of cell lines to graft. In this case we would need to use alternative patient derived cell lines. Moreover, if we receive more than the expected number of brain tumour cases, we could generate more cell lines for engraftment.

We may need to alter the dose of therapeutic agents and their delivery regimes to achieve maximum efficacy

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

Where possible, we will test multiple new treatment options simultaneously, so that control groups can be shared between experiments, reducing the total number of mice required. Our experimental design is based on previous work in animals, where we have shown that 6 animals per test group is sufficient to prove that a treatment is effective. We will maximise the amount of data we can acquire fromindividual mice by imaging them routinely using methods specific to each experiment. Moreover, once all relevant therapeutic data has been obtained, further information will be gained from these mice using electrophysiological monitoring under terminal anaesthesia. Whole mouse brains will be collected so that we can have matched tumour and non-tumour sides, allowing us to test the healthy tissue for comparison, without the need for another separate 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, we will collect other tissues such as the liver, spleen, lungs and bone marrow. This will allow us to either pass the tissue on to other groups for analysis in other projects, and to collect immune cells for culture. As we are using common mouse breeds, we will utilise mice that are surplus to requirements where possible.

Refinement

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

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

We will inject cancer cells into the brains of mice to establish a tumour. Unfortunately, there are no naturally occurring brain tumours in mice, so this route of developing brain tumours in mice is absolutely necessary and does give us complete control over tumour establishment. We can use imaging techniques to monitor the growth of tumours to make sure that they don't get too big. This will allow us to stop the experiment before any of the mice get too unwell. Therefore, they should not suffer any unnecessary pain or discomfort during the course of the experiment. Where appropriate, we will anaesthetise the animals so that they are not distressed whilst carrying out certain procedures, and we will also give them pain relief to make sure they are not suffering.

We will strive to only test treatments that have already proved to be safe in animals and have been fully tested in the laboratory setting. For brain monitoring we will perform surgery to expose the brain on mice that have established brain tumours only under terminal anaesthesia. We will then insert probes into the brain to monitor differences in electrical and chemical signals between normal brain and tumour. We will use different types of mice for specific experiments. In cases where we want to test drugs on human tumours, we will use mice that lack key parts of the immune system to do so otherwise these tumours will be rejected.

These mice are perfectly healthy as long as they are kept in sterile environments to minimise the risk of an infection. The latter is provided by properly trained staff at our institution. For experiments with mouse cells, we will use normal mice, which have a fully functional immune system and therefore are unlikely to get any infections. We will test different types of human brain cancer cells to see if out treatments work on different types of brain tumours to reflect the differences in patients tumours. The surgical procedures we are using are well established in the scientific community. They have already been appropriately refined, but we will consult with vets to ensure that we minimise the possible distress caused to all animals. We plan to use female mice only, since they are easier to house together and less likely to fight and harm each other. Although the incidence of brain tumours is slightly higher in men than in women, treatment options do not differ

between males and females so results generated using females can be transferable to males. Our study will focus mainly on adult glioblastoma (GBM) for which there are currently no known causes. In this regard findings in female mice will represent accurate insight for clinical trials. In addition, studies in the lab using GBM tumour cells derived from male and female GBM brain tumour patients have not revealed any differences in our experimental findings.

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

We require the full complexity of an adult, living creature to prove that our treatments are safe and effective, so that they can be put forward for clinical testing in humans. This is only achievable with mice or other mammals.

Mice are easy to work with and the procedures we will use are already well established in them so we know that they will work. It would not be possible to use terminally anaesthetised animals in most of our experiments, because we have to allow days or even weeks for tumours to grow, so that we can prove that our treatments work.

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

For surgical procedures, animals will be given pain relief where appropriate to mitigate any discomfort associated with the procedure. Animals will also be given fluids during long procedures to insure that they do not suffer from dehydration. After procedures involving anaestheisa, the animals will be allowed to recover in a warm chamber until they return to normal activity. After they have completely recovered, then will be returned to their normal housing. Animals will be regularly monitored by staff in the animal unit, as well as the researchers carrying out the experiments, so that they are checked at least once per day. Any animals that appear to be sick or show signs of excessive weight will be specifically check twice per day and killed if deemed necessary.

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

We will use the guidelines set by our establishment to ensure the experiments are designed and carried out in the most refined way possible. We will also consult technical documentation from the NC3R's website to ensure that we are as up-to-date as possible with regards to new practices and techniques. In particular, we will ensure that our experiments meet the criteria set out in "Responsibility in the use of animals in bioscience research", as well as ensuring that we adhere to the ARRIVE guidelines for reporting animal research.

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

Regular updates from the staff that run the animal units at our establishment will inform us of advances in the 3Rs. In addition, we will seek out updated information from the NC3Rs organisation to make sure that our experiments are carried out in line with their policies. Any minor changes to the 3Rs will be implemented immediately, assuming that they do

not breach the terms of the project licence. Any significant advances in the 3Rs requiring changes to the project licence will be added as soon as possible. All changes will be discussed with the personal licence holders working under the project licence, and standard operating procedures altered to reflect these changes.

14. Epithelial homeostasis and cancer

Project duration

5 years 0 months

Project purpose

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

Key words

Cancer, Early Detection, KRAS, Pancreas, Epithelial homeostasis

Animal types	Life stages
Mice	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?

Epithelial tissues act as protective barriers to the external environment. Epithelial homeostasis, which describes a balance between growth and death in a tissue, is vital for tissue health. One key aspect of epithelial health and homeostasis is the removal of spontaneously occurring genetically mutated cells from a healthy tissue. Retention of genetically mutated cells in the tissue would lead to a loss of barrier function and would initiate cancer. The main goal of this project is to uncover the molecular mechanisms underlying how healthy tissues eliminate genetically mutant cells and determine how these processes are deregulated during early tumorigenesis. Our current understanding of these processes is poorly described.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 detection of cancer is key to improving patient survival. Most cancers are easier to treat, and patient prognosis and quality of life improve greatly when cancer is detected earlier. Our research focus is to understand the cellular and molecular biology of early tumorigenesis. A better understanding of the early stages of tumour growth and the timing of when tumours become dangerous, will lead to the development of new and improved early detection tools. It will also directly inform new biomarker discovery, improve our ability to diagnose lethal versus non-lethal lesions, and distinguish cancer diagnosis from non-cancer related pathologies. Early detection of cancer will also directly impact upon drug discovery research by improving patient stratification and recruitment to clinical trials.

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

This project will generate new information in the biology of epithelial tumorigenesis, particularly on the interplay between genetic predisposition and tissue health. Moreover, we expect to gain insights into epithelial tissue mechanics and health, which will impact upon the field of epithelial morphogenesis.

Novel findings will lead to high impact publications and new grant funding. This project will also generate new experimental models/approaches of sporadic cancers, particularly of cancers of unmet clinical need such as pancreas and lung.

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

In the short-term, outputs such as new information and experimental models will benefit the cancer research and epithelial cell biology research communities. The clinical benefits of this work are likely to be long term; our principal hope is that by studying the early stages of disease, we may contribute to the identification of new biomarkers and the development of new and improved detection and diagnostic methods, which together will allow us to intervene at early stages of disease and improve prognosis.

Moreover, this work may identify candidate genes and candidate genetic pathways as novel therapeutic targets to treat human cancer in the future.

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

All knowledge generated during this project will be disseminated as peer-reviewed publications and presented at national/international conferences/scientific meetings. Data will be shared via open access journals and repositories. We will continue to actively engage with the general public via events within the University/local communities and via national cancer charities. Progress and successes in key milestones will be released via our establishment’s research pages, newsletters and social media.

Species and numbers of animals expected to be used

- Mice: 8931 mice - we will use adult males and females.

Predicted harms

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

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

We will use genetically altered mouse models of epithelial cancer and primarily adult life stages (including aged animals; e.g., > 20 weeks old). Mouse models recapitulate human disease accurately at the genetic, tissue and clinical level. Our research goals are to investigate early stages of sporadic cancers in adult tissues, i.e., how cells carrying genetic mutations override normal tissue homeostasis to drive disease. By using genetically altered mouse lines and Cre-lox technology, we will determine genetic predisposition in specific target tissues and unravel whether/how exogenous risk factors such as inflammation cause disease. All mouse lines used in this project will also express a reporter (e.g., fluorescent protein), which will allow us to tag and trace the genetically mutant cells in tissues over time.

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

Typically, we induce conditional gene expression/deletion in adult mice (e.g., 6–8-week-old) using inducible-Cre recombinases and under the control of a tissue-specific promoter, e.g., tamoxifen induced Pdx-1 CreERT in pancreas tissues. Expression of Cre recombinase also induces expression of a fluorescent protein (e.g., RFP) from the ROSA26 locus. We are interested in sporadic disease; therefore, we typically use one injection of low dose tamoxifen to induce transgene expression. Induced animals are aged to a defined end point (e.g., 7 or 35 days) before being killed by a Schedule I method.

Alternatively, we assign animals to a controlled feeding programme (e.g., high fat diet for 3 months), or induce tissue inflammation (e.g., scheduled intraperitoneal injection of pro-inflammatory agent) before inducing conditional gene expression and ageing animals to a defined experimental time point (e.g., 35 days post induction of Cre).

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

We expect that expression of oncogenes such as oncogenic Kras in healthy adult tissues will lead to the development of premalignant lesions that will not have adverse effects on the animal.

Tumorigenesis requires the presence of a second mutation (e.g., loss of tumour suppressor function (p53) or expression of oncogenic c-Myc), or an exogenous risk factor such as chronic inflammation. In these cases, aged animals (e.g., > 100 days post induction of Cre) may experience the adverse effects of malignant tumours such as weight loss, hunching, inappetance, lack of grooming. Additional clinical signs of pancreas tumours may include weight loss from the haunches, abdominal distension, pallor in the extremities (e.g., paling feet). Lung tumours may cause breathing difficulties. Tissue inflammation alone may cause transient minor discomfort. Since we are interested in early tumorigenesis, we will not routinely use tumour burden as an end point and experiments will be stopped before the appearance of clinical signs.

Clinical Signs Health Score sheet for this project is inserted here:

Score Sheet Guidance

A score sheet combines general health indicators with expected adverse effects from your PPL so that Animal Technicians and NACWOs can ensure the highest standards of animal care and welfare, even if they are unfamiliar with this research/strain. It offers clear instructions about the appropriate actions to take at varying stages of sickness/expected adverse effects to safeguard appropriate care.

General health indicators (Wolfensohn and Lloyd)

Parameter	Animal ID	Score	Date/Time	Date/Time
Appearance	Normal	0		
	General lack of grooming	1		
	Staring coat, ocular, nasal discharges	2 each		
	Piloerection, hunched up	4 each		
Weight Loss	Normal	0		
	5% weight loss	1		
	Up to 15% weight loss	2		
	Over 15% weight loss	4		
Natural Behaviour	Normal	0		
	Minor changes e.g. Lack of nest	1		
	Less mobile and alert, isolated	2		
	Vocalisation, self-mutilation, restless or inactive, cold	4 each		
Provoked Behaviour	Normal	0		
	Less active and/or slow movement	1		
	Only moves when directly provoked	4		
	Does not move, even when provoked or displays hyperactivity when provoked	9		

Expected adverse effects for pancreatic cancer or lung cancer models:

Parameter	Animal ID	Score	Date/Time	Date/Time
Appearance	Normal	0		
	General lack of grooming	1		
	Staring coat; ocular, nasal discharges; pallor of eyes, nose, feet; abdominal distension.	2 each		
	Pallor of eyes, nose, ears and feet; Intermittent diarrhoea.	3 each		
	Piloerection, hunched up; complete paling of extremities & animals feel cold; continuous diarrhoea (>72h); abdominal distension affecting breathing or movement	4 each		
Weight Loss	Normal	0		
	5% weight loss	1		
	10% weight loss	2		
	Up to 20% weight loss, particularly at haunches.	4		

	Over 20% weight loss, particularly emaciated at haunches with clear distension of spine/pelvic bones or pelvic bones are readily palpable.	8		
Natural Behaviour	Normal	0		
	Minor changes e.g. Lack of nest, inappetence. Shallow or irregular breathing.	1		
	Less mobile and alert, isolated, reluctance to move; Altered respiration, temporary or intermittent abnormal breathing pattern, Intermittent 'chattering.	2		
	Vocalisation, self-mutilation, restless or inactive, cold; Laboured respiration, prolonged 'chattering'.	4 each		
Provoked Behaviour	Normal	0		
	Less active and/or slow movement	1		
	Only moves when directly provoked	4		
	Does not move, unresponsive, even when provoked or displays hyperactivity when provoked	9		

Parameter	Animal ID	Score	Date/Time	Date/Time
Tumour Phenotypes	Small skin papilloma <0.1 cm.	2		
	Tumour mass <1.5 cm; skin papilloma <0.5 cm	4 each		
	Tumour mass >1.5 cm; ulceration; skin papilloma >0.5 cm.	8 each		
Post-surgical wound; Wound healing	Normal	0		
	Slight redness, clear fluid, scratching/licking	1 each		
	Missing suture/clip, redness, inflammation	2 each		
	Wound shows evidence of herniation/rupture or over 7 days without healing	4		
		Total:		

Score Key

Score	
0	Normal
1-3	Use observation card
4-9	Monitor carefully, seek advice from NACWO
9-12	Pancreatic cancer mice: kill by an appropriate method. Non-pancreatic cancer mice: Observe regularly, NACWO must be involved to discuss whether endpoints have been reached. ***Advice may need to be sought from the NVS regarding ongoing treatment e.g. antibiotics, analgesia, fluid therapy***
13-15	Kill by an appropriate method

Note: The clinical signs outlined in the score sheet will be used to assess the health of experimental animals. However, the judgement and experience of the licensee may necessitate actions at earlier time points, as appropriate. **Clear humane endpoints are detailed in each 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)?

We expect tumour development to have moderate severity. Only animals that carry site-specific Cre recombinases and a combination of conditional alleles will develop tumours.

Expression of oncogenic Kras in healthy adult tissues will not lead to tumour development. Tumorigenesis requires the presence of a second mutation (e.g., loss of

tumour suppressor function such as p53 or expression of oncogenic c-Myc), or an exogenous risk factor such as chronic inflammation. Based on current experimental design, approximately 25% of cohorts may 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?

Mouse models of cancer are clinically relevant and accurately recapitulate the full spectra of human disease at the genetic, cellular and tissue level. Cancer is multifaceted disease that starts with genetic mutation in cells; however, for tumours to progress to malignancy mutant cells 'hijack' normal biological processes (e.g., immune cell infiltrate, stromal cells, extracellular matrix modification) to support tumour progression. Mouse models accurately model all aspects of tumour complexity that would be technically challenging to achieve using ex vivo or in vitro cell systems alone.

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

Drosophila melanogaster and ex vivo organoid models may be used as non-animal alternatives for some aspects of this work e.g., a reductive approach to unravel molecular cell biology of a phenomenon at the single cell level. Organoids also allow for high-throughput experimental approaches, e.g., testing efficacy of pharmacological inhibitor or oncolytic virus before administering to animals. However, these systems cannot replace the need for animals in this project.

Why were they not suitable?

Drosophila melanogaster as a model of human cancer is hugely limited as it does not fully recapitulate mammalian/human tissue complexity and physiology. Pertinent to this project is the fact that insect models lack a distinct pancreas and lung tissue, and an adaptive immune system. Ex vivo pancreas and lung organoid models do not robustly recapitulate healthy normal tissue, principally because both pancreas and lung tissues are complex, composed of multiple cell types; are not derived from a distinct stem cell pool; are highly plastic and readily reprogramme following mutational insult and injury. Moreover, we lack capabilities to model all aspects of tissue inflammation or diabetes ex vivo.

Reduction

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

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

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

NC3Rs Experimental Design Assistant and Power Calculations. Effect size (m1-m2) and Variance (S.D.) were calculated using current data.

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

All calculations were made using the NC3Rs Experimental Design Assistant and current data.

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 phenotypes require transgene heterozygosity; therefore, we will breed heterozygous pairs/trios to increase efficiency. We generally divide target tissues (e.g., pancreas) into multiple fixatives (e.g., OCT versus FFPE) and experimental pipelines in order to maximise the output of each induced animal. In future studies, we plan to use mathematical in silico modelling and collaborative projects to address specific questions, which will reduce the requirement for animals. Where specific signalling pathways are to be interrogated using pharmacological inhibitors, we will use pilot studies to gauge efficacy (e.g., 2-4 animals). We will also make available and share tissue to other projects if appropriate. Wherever possible, we will use imaging techniques (e.g., PET scanning) and/or microsampling of blood to measure longitudinally and maximise experimental outputs on a minimal number of animals.

Refinement

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

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

We will use genetically altered mouse models and site-specific Cre-lox technology that allows temporal and inducible gene modification in specific epithelial tissues. Mouse strains will also express reporters of Cre that will allow tracing of recombined (i.e., genetically mutant) cells in tissues over time. All genetically altered lines will carry conditional alleles; therefore, only mice that carry site-specific recombinase and the conditional allele will develop tumours. These models are well characterised by our collaborators; experimental time points and timelines of the development of predictable tumour phenotypes is known. We will only use genetically altered animals appropriate to

our objectives (i.e., targeting specific transgenes in a tissue-dependent manner) to ensure that the work carried out is accurate. Our primary research focus is to understand early tumorigenesis; therefore, we will not routinely age tumour-bearing mice or use tumour burden as an end point. Moreover, we routinely model sporadic disease, which requires minimum procedural intervention. In vivo imaging approaches (where appropriate) provide non-invasive methods to monitor tumour progression in the same animal over time.

Controlled feeding regimes will allow us to generate in vivo models of obesity and diabetes without genetic alteration and extensive breeding. Tissue transplantation assays provide approaches where we can uncouple cell intrinsic from cell extrinsic factors in vivo; test putative cancer stem cells and monitor metastasis in short-term assays without the need for primary tumour formation.

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

We are primarily interested in Kras-driven epithelial tumours e.g., pancreas and lung. Both pancreatic and lung cancers are adult diseases that have increased risk in ageing populations. Therefore, immature life stages are not appropriate for our research studies. Moreover, developing epithelial tissues are highly plastic, morphogenetic structures that do not recapitulate fully differentiated adult tissues. Moreover, tissue health requires functional tissue dynamics and mechanics, and architecture; all of which cannot be modelled in terminally anaesthetised animals. Less sentient species such as Drosophila melanogaster are good alternatives for some aspects of this work and will be considered where appropriate; however, Drosophila models do not have an adaptive immune system, or distinct pancreas/lung tissues.

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

Refinements will include frequent monitoring of induced animals, post-operative or anaesthetised animals. Exogenous substances/agents will be administered at the minimum dose, as determined using NC3Rs guidelines for best practice and dosing regimens and following consultation with our collaborators.

All procedures will be carried out by trained and experienced staff with the support of experienced animal facility staff/NACWOs/NVS (e.g., application of anaesthesia, analgesics, surgical techniques). Clear humane end points are detailed for each protocol. Animals will be housed in clean, pathogen free environments to ensure good animal health is maintained and the risk of infection from the environment is minimised.

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

This project will be carried out in collaboration with experienced cancer researchers who have excellent track records and are world renowned leaders both in the pancreatic and lung cancer fields, and in the use of genetically altered mouse models of disease.

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

Engage with NC3Rs representatives on a regular basis via local workshops/meetings, newsletters and social media.

15. Peripheral Nerve Regeneration

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

peripheral nerve injury, stem cells, biomaterials, surgery

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 improve peripheral nerve regeneration following traumatic injury using stem cells and biomaterials.

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

Why is it important to undertake this work?

Peripheral nerves carry information from the brain and spinal cord to their target organs. They are essential for movement, sensation and other important functions such as temperature control.

Peripheral nerves are commonly injured following trauma (e.g road traffic accidents, workplace incidents) or following surgical removal of a cancer (e.g. prostate cancer). The

subsequent disability following peripheral nerve injury can be significant and wide ranging effects including (but not limited to) complete loss of a limb's function, sexual dysfunction, inability to control and move facial muscles and pain. Current treatment options are limited and even with optimal access to current treatments, outcomes remain extremely poor.

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

The primary output of this project is to produce a medical device that (i) replaces the need for a nerve graft (where a healthy nerve is sacrificed in order to reconstruct an injured nerve) and (ii) improves upon outcomes for patients receiving this treatment. Outputs will also include scientific publication of our findings which will inform further research in the field.

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

Within the short term, researchers looking at further understanding the nature of peripheral nerve injury will be greater informed by the publication of our findings. In the long term, if successful, patients with peripheral nerve injury will benefit from an alternative treatment options to nerve grafting (which otherwise involves sacrificing the function of a working nerve).

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

All work, unsuccessful or successful will be submitted for publication. This will prevent duplication of work by other groups and guide further research direction. Our work is a collaboration between two groups with extensive experience in biomaterials and peripheral nerve, by combining expertise we increase the chance of success.

Species and numbers of animals expected to be used

- Rats: 300

Predicted harms

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

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

We have chosen to conduct the experiments described on adult rats for the following reasons:

- We have considerable expertise evaluating a similar medical device in rats, and taking this treatment into human trials. This expertise allows us to better plan the experiments required, such that only a minimum number of animals are required.
- The rat sciatic nerve model is similar in size to a human digital nerve and it is therefore pragmatic for suturing biomaterial conduit interventions.
- The rat model of sciatic nerve injury is used by many other researchers internationally and the outcome measures are well documented; therefore by utilising a well established model, it is easier for our research to be appropriately scrutinised and compared to other findings.

- Additionally, there is less need for other groups to duplicate this research, thereby reducing the overall need for further animal research.
- Rats undergo nerve regeneration at a fast rate. Therefore we can assess the outcomes of the experiment in less time, hence minimising animal suffering.
- Immature rats have an even greater capacity for regeneration, such that this may limit the ability to accurately evaluate the benefit of the experimental treatment. In addition, the nerves that we would seek to reconstruct are much smaller and the repair would be less representative of a human repair, nor would the creation of an appropriate sized defect be possible.

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

Rats will undergo a surgical procedure whereby a nerve to one leg will be cut and then repaired through a variety of methods, including the experimental treatments we are investigating. The majority of rats will then not require a further surgical procedure. They will be monitored closely to support their welfare to the time points already listed. During this time, behavioural studies will also be conducted. For the majority of animals, at set time points, they will be humanely culled and their tissues closely examined to obtain the experimental outcomes. For a subset of rats, they will undergo a further general anaesthetic to allow testing of the nerves function. These rats will be humanely culled prior to recovery from anaesthetic.

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

Animals are expected to experience mild postoperative pain in the short term and this will be ameliorated with pain-killers. There is likely to be some reduction in function to the limb with the nerve injury. In our experience this does not seem to effect the rats mobility or cause any signs of distress.

In the long-term models (3-6 months), animals could undergo autotomy, where the rat chews the nails of the numb hindpaw. We will mitigate this by housing in groups with a calm environment and the use of strains not thought to exhibit autotomy and, therefore, expect autotomy to be less than 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)?

All animals are expected to experience moderate severity outcomes due to them all undergoing a surgical procedure resulting in reduction in the function of one limb

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 created an artificial nerve graft that has been tested successfully in the laboratory but prior to testing in a human would need further evaluation in an animal to ensure safety and effectiveness of its use in a similar environment to those injuries seen in humans. Human injuries tend not to be standardised and it is very difficult to control for a multitude of variables as is possible in an animal model.

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

We have tested materials in the laboratory cell culture models with nerve cells and stem cells to choose the material most likely to be successful prior to animal trials. Moving forward from here, animal studies are an essential next stage to ensure safety before considering placing these devices in humans.

Why were they not suitable?

Laboratory testing of materials to assess nerve cell and stem cell growth has been considered suitable and carried out, however, this information is not sufficient to move toward human trials. Animal studies would be required prior to human studies to maximise human safety.

Reduction

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

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

Within each experimental group we will use 8 rats. There are 4 experimental groups. Each timepoint will require 32 rats. We estimate utilising at least 8 time points.

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

Only one material hydrogel is being taken forward via this project as other potential materials were tested using non-animal laboratory techniques thereby reducing the number of animals required. Full review of the published literature has been undertaken to ensure that there is not undue duplication of experimentation.

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

Close monitoring of animal welfare to reduce the risk of loss animal loss.

Any adjustments to materials considered would be tested in the laboratory before testing 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.

From our experience gained over the past years using these animal models, it is apparent that the experimental procedures described in this application do not greatly affect the well being of the animals. The majority of described procedures are carried out whilst the animals are fully anaesthetised. The animals do not appear to have persistent adverse effects from the anaesthesia. From past experience, after the nerve injury, the animals do not show any behavioural signs of pain whether the nerve repair is carried out immediately or with delay. Nerve injury with/without repair can partially affect the gait, but has little effect on the mobility of the animals. The insertion of the nerve conduit at the site of repair does not affect the mobility of the animal, because the biomaterial is soft and flexible, and after harvest histology, showed no signs of adhesion or inflammation in past experiments.

In the long-term models (3-6 months), animals could undergo autotomy. This will be mitigated by using strains of animals that are not expected to show autotomy and using refined animal care strategies.

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

The intervention requires suturing of nerve endings to a device. At a more immature life stage repair is more difficult and less likely to be representative of the repair used in humans. Additionally, at a non- adult stage, the ability to self-regenerate nerve is much greater, and therefore would not accurately evaluate the efficacy of the device being tested. Recovery of function secondary to the intervention is an important experimental outcome therefore utilising only terminally anaesthetised animals would not allow this to be observed.

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

All animals will be monitored closely for any signs of pain or distress, especially in the immediate post- operative period. Analgesia will be provided where necessary and consultation with the NVO if there are other concerns.

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

We will follow best practice guidance published by the National Centre for Replacement, Refinement and Reduction.

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

The investigators are subscribed to updates from the National Centre for the Replacement, Reduction and Refinement of Animals in Research and will implement any advances in a timely manner.

16. Genetic and physical control of B cell and antibody responses

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

B cell, Antibody response

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 main goal of this research is to understand how B lymphocytes, the white blood cells that produce antibodies, detect and respond to infections and vaccines, and why they sometimes fail to protect or even cause an immune 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?

Antibodies are essential to our immunity. They provide long lasting protection from a wide variety of infections and are responsible for the success of most vaccines. Problems in antibody production often cause immune deficiencies. Therefore, there is an ongoing interest to improve antibody production through vaccine design, and thus to combat new and highly dangerous pathogens.

On the other hand, production of antibodies that is too high can cause autoimmunity and allergy, and the cells that produce antibodies also give rise to leukemias and lymphomas.

All of these diseases are on the rise in modern society. Animal models help us to understand the genetic and physical mechanisms that underlie a balanced antibody response and provide insights into human disease.

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

The primary output of this work will be new information on regulation of antibody responses. We will determine where and for how long vaccine antigens remain in the body and how this impacts on antibody production. We will also identify specific genes acting in antibody production from within B cells. This new information will inform vaccine design and advance our understanding of immune diseases, such as immune deficiencies, autoimmunity and allergy. We will publish our findings in peer- reviewed scientific literature, freely accessible using open access. We will also immediately share preprint articles in publicly available on-line depositories, such as BiorXiv. We will summarise our overall findings for students and trainees in reviews and in viewpoint articles, including articles for the lay public.

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

Our results will be of immediate interest to a broad range of academics interested in antibody responses, B cell biology, B cell pathology, allergology, and in the role of stromal cells in immunity. The outputs will include both new information as well as new mouse lines generated in this programme. In the longer term, new information gained in our studies will be relevant to clinical researchers and the pharmacological industry designing vaccines, and therapies for patients with antibody deficiencies, autoimmunity, allergy and lymphoma.

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

In addition to publications, we will disseminate raw datasets through publicly accessible databases, including negative findings. Depending on our results, our future plan is to collaborate with clinical researchers to follow up on our findings and study patients with autoimmunity, immune deficiency and severe allergy using established cohorts at our collaborator's sites. We will also pursuit emerging therapeutic options, for example relevant to human allergy, drawing on experience of our current collaborators.

Species and numbers of animals expected to be used

- Mice: 19500

Predicted harms

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

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

The immune response cannot be fully recapitulated outside of a living animal. Mice are the least sentient animals with an immune system resembling that of humans. There is an enormous amount of tools available to study the mouse immune system, genetic alterations are well developed and a large number of mutant strains are available.

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

Genetically modified mice will be bred to obtain mutations specifically affecting cells of the immune system. These animals along with wild type controls will undergo manipulation of the immune system, such as bone marrow transplantation, or receive substances modulating immune cells. Typically, the mice will then be immunised with vaccine-like materials to study the antibody response. Some animals will be exposed to allergens to induce allergy.

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

Most of our procedures, including breeding, and immune challenge are expected to be of mild phenotype with only a fraction reaching to moderate signs. Immune deficiency due to genetic defects or bone marrow transplantation is typically well tolerated by the animals in specific pathogen free conditions and by supplementation of drinking water with antibiotics. Animals challenged with model vaccines are expected to exhibit only transient discomfort and no lasting harm. Genetic perturbations causing immune disease or lymphoma only manifest in animals aged beyond the duration of our experiments. Exposure to allergens induces asthma-like changes in the respiratory system, which are typically well tolerated.

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

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

We expect that in total, approximately 93% of the animals will experience subthreshold or mild severity. Approximately 7% will reach moderate 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?

The reason for using animal models for our experiments is that the development of the cells of the immune system and of the immune stroma, and their complex interactions cannot be fully recapitulated outside of the animal. In addition, disease conditions associated with immune system pathology develop as a result of imbalance between factors that are impossible to reconstruct in vitro.

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

In vitro assays for cell function, immune cell co-culture and organoid experiments.

Why were they not suitable?

While in vitro assays are informative for formation of hypothesis about B cell functions, they do not currently fully capture the complex mechanisms regulating their interactions with antigens and with the immune environment that underlie the immune response. There are currently no in vitro systems that recapitulate the correct architecture and cellular composition of the immune stroma.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 represents the sum of the animals needed for the experiments and the numbers of wild type and genetically modified breeders needed for their generation.

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

Our experiments use robust statistical determination of the minimal possible numbers of animals to use in the experiments as recommended by the NC3R. We have also implemented cell transfers and modern single-cell analysis techniques that dramatically reduce the number of animals without compromising the quality of the 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 are working within institutes where sharing and carefully organised breeding reduces the overall numbers of animals used. Strains not in use will be cryopreserved.

Refinement

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

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

Which animal models and methods 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 mouse models of the immune response. Mouse immune system, including the antibody response, has been extensively studied and closely resembles the human immune system. An enormous amount of reagents is available for studying of the mouse immune system, and mouse transgenic and knockout techniques are well established, with mutant strains widely available. This allows us to breed animals in such a way so as to generate mutations only in specific cell types, or inducible pharmacologically. Although this increases the numbers of breeding animals, it limits harmful genetic effects these mutations may have in other cells of the body. In addition, there is extensive previous experience with animal procedures used in this project. This allows us to carry out experiments efficiently and with good knowledge of possible harms.

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

To our knowledge the laboratory mouse is the least sentient species that fulfils the requirements of this research. It has a well developed immune system that resembles that of humans. Lesser sentient animal models do not have similarly developed lymphoid organs and do not produce similarly functioning humoral response or pathology.

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

The proposed procedures have already been refined based on current best practices. Any new developments in animal welfare or scientific improvements will be implemented based on recommendations of our veterinary surgeon.

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

We will be following current accepted guidelines, the ARRIVE guidelines (Kilkenny et al, 2009) and the PREPARE guidelines (Smith et al, 2017). Our institute also issues a regular newsletter which contains relevant updates and we will implement any advancements published in the scientific literature related to the project.

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

The PI and staff working on the project will attend regular refresher seminars on animal welfare and will undergo competency re-training.

17. Preventing muscle wasting in kidney failure

Project duration

3 years 0 months

Project purpose

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

Key words

Chronic Kidney Disease, Glucocorticoids, Muscle Wasting, 11Beta Hydroxysteroid Dehydrogenase

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To identify how kidney disease leads to muscle wasting and identify novel treatments to prevent this complication.

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

Why is it important to undertake this work?

Chronic kidney disease (CKD) is characterised by persistent Reduction in kidney function and associated harms to the circulatory, musculoskeletal and immune systems. In the UK, an estimated 6.5 million people live with CKD. Half of these develop muscle wasting and fatigue as a result of their kidney disease. For patients with kidney failure and muscle wasting, the risk of death is 3-fold higher than for patients without muscle wasting. In addition, poor muscle health is linked with increased risk for bone fractures, heart attacks and strokes, admission to hospital and worse quality of life. Therefore, muscle wasting

related to CKD puts a significant burden on individuals, as well as health and social care services. Unfortunately, the causes for muscle wasting in CKD are poorly understood, and there remains a lack of effective medical treatments for managing these patients. This has been recognised in national and international research strategies, where studies designed to understand and prevent muscle wasting in CKD are a priority.

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

This project will provide new knowledge on the process of muscle wasting in patients with chronic kidney disease. This knowledge will be shared with the wider research community in the form of publications in scientific journals and presentations at conferences. Furthermore, the project will characterise putative therapeutic targets for the prevention and treatment of muscle wasting. These data will inform the development of new drugs and clinical trials with the goal to help patients with CKD and muscle wasting.

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

In the short-term, the improved understanding of the processes that underpin muscle wasting in kidney disease will benefit many other research groups exploring muscle wasting in human disease. This will include fundamental insights about the origins of muscle disease that are applicable in other conditions (e.g. acidosis or insulin resistance as drivers of muscle wasting are also important factors in diabetes, respiratory failure or critical illness). These findings will be shared with the research community over the next 1-3 years of the project.

In the medium-term, pre-clinical data on 11bHSD1 inhibition as a therapeutic target in muscle wasting and kidney disease will benefit investigators planning clinical trials. It will guide the design of future clinical trials against muscle wasting in kidney disease, in particular whether 11bHSD1 inhibitors hold promise in this condition. Furthermore, 11bHSD1 inhibitors are being explored in a wide range of other

clinical settings, where our data will be valuable to guide general safety and efficacy considerations. These benefits of our research are expected to manifest in 5-8 years.

In the long-term, the development of novel treatment approaches to prevent muscle wasting in patients with chronic kidney disease would mean that patients with this condition are the major future beneficiary of this research. The clinical benefits may be transferrable to other patient groups with muscle loss unrelated to kidney disease, who share common underlying pathophysiology. Reducing muscle loss in people with comorbidity holds the potential to improve general health, wellbeing, reduce care needs and reduce mortality. These benefits may take 8 to 10 years or longer to realise.

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

This research fits within the interest of major research centres of excellence studying musculoskeletal aging. Through our current links and involvement with these centres, we would ensure wider dissemination of findings to research organisations working in this field, as well as interested patient engagement groups. All findings will be routinely

published to inform methodological approaches in appropriate journals and academic conferences regardless of experimental outcome.

Species and numbers of animals expected to be used

- Mice: 1750 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.

Studying diseases in mouse has contributed to valuable scientific and medical advances for relieving kidney disease and its complications in humans. Mouse bodies share sufficient similarities with human body to permit meaningful scientific study of kidney disease and associated muscle wasting. The requisite scientific methods are well established for mice, meaning that further experiments on animals to develop reliable methods can be kept to a minimum. Exploring novel treatments on animals before humans reduces risks of harm to humans.

The study will use genetically altered animals. Producing and maintaining these animals will employ breeding techniques that involve all stages of the life cycle.

Research on kidney disease and associated muscle wasting requires that animals have completed development and will therefore use adult mice only.

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

Breeding genetically altered animals involves mating and reproduction of mice by natural means. Small tissue samples may be taken infrequently to track the genetic alterations present in each animal.

Adult mice will receive a modified diet (adenine supplementation) which will lead to slowly progressive Reduction of kidney function over several weeks. During the experiment, mice may undergo blood tests, injections of insulin or glucose, testing of grip strength and short-term single housing for metabolic monitoring. Further samples and data will be collected during non-recovery general anaesthesia or after death.

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

Maintaining genetically altered animals does not normally cause significant or lasting discomfort for animals. Animals will be monitored regularly. If an animal is observed to have persistent discomfort beyond mild changes in appearance or behaviour, it will be removed from the experiment at this stage.

Reduced kidney function does not cause pain for animals, but inevitably causes lower food intake and associated weight loss. These effects are expected to become more

pronounced the longer the animal is maintained on the altered diet. The maximum duration for an animal to receive the altered diet is 7 weeks. Body weights of animals on modified diet will be monitored regularly and animals will be observed for altered behaviour. If an animal loses weight rapidly, stops eating or displays persistent abnormal behaviour, it will be removed from the experiment and steps taken to end suffering.

Injections, blood sampling, grip strength measurements and short-term single housing for metabolic monitoring may cause mild discomfort that is short-lasting. In exceptional circumstances when more significant discomfort is observed in an animal with these procedures, it will be removed from the experiment and steps taken to end suffering.

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

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

All animals receiving an altered diet to induce kidney dysfunction are expected to experience a moderate level of weight loss.

The majority of animals undergoing breeding procedures, injections, blood sampling, grip strength testing or short-term single housing may experience short-lasting and mild 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?

Muscle wasting in kidney failure is a complex multi-organ disease process. Animal studies are essential to clarify the role of whole body and muscle-specific glucocorticoid metabolism in this condition. Feasibility of cause-effect studies in humans is limited by safety considerations and cost. Whilst cell culture experiments can replicate some kidney failure conditions and provide knowledge on cellular and molecular processes, these types of laboratory studies are inadequate to replicate all the complex interactions that take place in a living organism. Animal studies are needed to confirm the observations from cell culture studies and explore whether new treatments that block glucocorticoid metabolism are effective against muscle wasting in kidney failure. Testing this intervention in animals prior to clinical trials is an ethical obligation and will reduce potential harms for humans.

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

We have considered analysing existing scientific datasets, conducting studies in humans and using cell culture models of muscle tissue.

Why were they not suitable?

Analysis of existing datasets: We have already analysed existing datasets from observational studies in patients with kidney disease or from clinical trials of 11bHSD1 inhibitors in patients without kidney disease. While the results are generally supportive, these datasets are not sufficiently detailed or applicable to allow a definite answer to our research questions.

Conducting studies in humans: We will perform an observational study in humans with kidney disease looking at clinical signs and muscle tissue biopsies. This will provide valuable information to support and confirm findings from animal research. However, the observational study design will not allow us to prove cause-effect relationships or prove the effectiveness of new treatments. The risks associated with conducting experimental or clinical studies in humans with kidney disease cannot be justified at this stage, without first obtaining further basic scientific knowledge and proof of concept from animal research.

Cell culture models of muscle tissue: We will use cell culture models wherever possible to study cellular and molecular processes and reduce the number of animals required for research. However, tissue culture models cannot adequately replicate the complex interactions between multiple organ systems that occurs in kidney disease in a living organism. Moreover, tissue culture models cannot adequately replicate the specific environment of physical forces that act on muscle during movement. Animal models are therefore needed to replicate human disease faithfully and address our research question with relevance to clinical medicine.

Reduction

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

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

Colony sizes for breeding animals have been determined based on existing breeding protocols for these transgenic mouse models that are ongoing within our Establishment. These are under continual review with expert advice from both the NACWO and NVS to ensure that numbers required for maintenance and breeding new transgenic lines are kept to a minimum. Experimental animal numbers are informed by current related active projects working with 11bHSD1 KO mice and through consultation with collaborators experienced in the adenine diet induced model of chronic kidney disease. The scientific literature has been reviewed to inform how much change in muscle mass is expected to occur in these animals and how many animals will be needed per experimental group to reliably detect this change.

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

We selected a diet-based model for kidney disease that is more reliable, consistent and has lower mortality compared to alternative surgical models. This reduces variability in experimental outcomes and reduces the number of experimental animals required to obtain meaningful results.

The experiment is devised in a 2x3 factorial design, allowing efficient use of data generated from control groups and thereby minimising the number of animals required.

We have conducted a thorough literature search and consultations with expert collaborators to inform statistical calculations on the lowest required number of animals to detect meaningful scientific results.

The planned pilot study adheres to a design that is embedded in the experimental protocol. This means that data from animals used in the pilot study can contribute to final result analysis, rather than duplicating use of animals.

Care has been taken throughout the experimental design and with selection of outcome measures to reduce unwarranted variability as much as possible. Steps to this effect include randomisation, blinding, or selecting muscle mass as a highly sensitive outcome measure. This will reduce the number of animals required to identify 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?

In house resources and expertise will reduce the number of animals needed to deliver this project. C57Bl6 mice with appropriate genetic alterations for this protocol are already available in house, reducing the need to establish new genetic lines or backcrossing animals. Breeding practises will adhere to local standard operating procedures and national NC3Rs guidance to maximise efficiency.

Our research group has previously optimised the data and tissue collection methods required to deliver this project. This avoids need for further optimisation, reduces variability and ensures that high quality data is obtained from every animal. Where operator-dependent measurement variation is possible, we will take precautions to perform measurements according to standard protocols and by the same operator wherever feasible. The use of tissue from each animal will be maximised by sampling all available muscles for subsequent analysis. In addition, primary muscle cell cultures will be derived to further extend material available for molecular analysis.

Finally, we will conduct a pilot study to confirm that our estimates for the minimum required animal number is indeed correct, prior to embarking on the full-scale project.

Refinement

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

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

Mice with genetic alterations in 11bHSD1 will be used. These animals display no apparent abnormalities or signs of harm.

Mice will receive dietary supplementation with adenine to induce gradual persistent Reduction in kidney function and associated changes like muscle wasting. These changes imitate the disease process of chronic kidney disease in humans.

The effects of adenine on the kidney are controlled by the dose and duration of adenine exposure. This allows for fine-tuning the level of kidney impairment to the minimum required for the planned experiments, thereby avoiding undue harm. Furthermore, it means that changes for the animal are mild early on, building only gradually over time. Hence, the animal experiences more significant changes only for a limited duration in the late phase of the experiment. Effects of adenine are very specific on the kidney, as it is concentrated there. Direct harm to other organs such as muscle or liver is avoided.

A diet-based model is technically much simpler and more reliable than alternative surgical models. This pre-empts animal harms and losses related to procedural complications or variation in operators' skill level. Furthermore, it spares animals from distress of anaesthesia and traumatic recovery periods.

To collect scientific data, animals will experience blood sampling, strength testing or a short period of isolation housing. Adverse effects related to these procedures are usually only mild and transient. The procedures will be conducted in the least invasive fashion (e.g. blood micro-sampling) and utilise species-appropriate behaviours (e.g. holding onto a grip bar).

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

The research project examines dynamic interactions between organ systems, namely kidney and skeletal muscle, in the setting of persistently reduced kidney function. Lesser developed species (e.g.

invertebrates) or aquatic life forms do not possess kidneys that resemble human kidneys and are therefore not a suitable model. To accurately model the changes in muscle associated with long-term kidney impairment, animals need to be studied over an extended period of time that precludes experiments on immature life stages or animals under terminal anaesthesia.

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

We reviewed the scientific literature extensively for mouse models of kidney disease and developed a collaboration experts in this field. This has allowed us to devise a purpose-specific protocol that allows us to study muscle wasting in kidney disease, while minimising harm to animals. In addition to employing an evidence-based protocol, we will

conduct a pilot study to confirm the validity and tolerance of the adenine-diet protocol prior to proceeding to the full-scale experiment. This will introduce a distinct step at which further Refinement can take place.

Our protocol includes provisions to track changes with kidney function (serum creatinine and urea) and changes in muscle function (grip strength testing) over time. Furthermore, we will test kidney function in a direct and more accurate fashion than blood tests alone (creatinine clearance). This data will help to ensure that the animal model is not progressed beyond the least harmful stage required to address the research question.

Suitable adjustments will be implemented in the care and housing of animals to mitigate adverse effects that may occur. Water and food will be provided in the most attractive and convenient fashion for animals to avoid dehydration or weight loss. Body weight will be monitored regularly and animals will be removed from experiments if they are at risk of severe malnutrition. Animals will be habituated to handling and test procedures to reduce associated distress. We will follow refined handling techniques as recommended by NC3Rs wherever possible (e.g. handling tunnels). If pain is suspected, appropriate analgesia will be provided.

Throughout the experiment, animals will be carefully monitored and welfare will be tracked using a multi-component score sheet. If features of undue adverse effects are observed in an animal, that animal will be removed from the experiment to relieve suffering.

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

All experiments will abide by local best practice procedures for breeding, maintenance and surveillance of animals. National guidance by the Laboratory Animal Science Association, National Centre for the Replacement, Reduction and Refinement of Animal Research, and the Royal Society for the Prevention of Cruelty to Animals was used in the development of this protocol, including:

Avoiding mortality in animal research and testing (https://view.pagetiger.com/RSPCAAvoidingMortalityResearchReport/RSPCA)

Blood sampling techniques, frequencies and volumes (https://nc3rs.org.uk/3rs-resources/blood- sampling, https://www.nc3rs.org.uk/blood-sample-volumes)

Genetically altered mice (https://nc3rs.org.uk/gamice)

Animal handling and husbandry (https://nc3rs.org.uk/3rs-resources)

The PREPARE guidelines (https://norecopa.no/prepare) informed the project planning phase, including engagement of relevant stakeholders and implementation of quality controls. Furthermore, the ARRIVE guidelines have been considered to ensure that results can be communicated with the highest scientific rigour.

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

We will remain in close dialogue with all staff in the care and welfare of animals including the NVS and the NACWOs to extend our knowledge about general and specific welfare issues relating to the experimental animals. We follow published methods to refine animal welfare in the polyarthritis models, attesting our commitment to continuous development of our research methodologies. Furthermore, we are working closely with other national centres for animal models of kidney disease to exchange knowledge advances efficiently. These efforts will ensure that we continuously up to date on the highest welfare standards specifically relating to our animal models and promptly refine procedures wherever possible.

In a dedicated effort to advance on the 3Rs, we will conduct a pilot experiment with 3 experimental and 3 control animals. This will ensure that our protocols reach the desired experimental targets, without leading to undue or unexpected harm to animals. This will include validation and Refinement for the animal welfare monitoring protocol and adjustments to mitigate potential harms or suffering.

Our group is actively engaged in research to replace and reduce animal research projects in the future. We are conducting research to validate and extend the use of muscle cell cultures for the investigation of muscle changes in kidney disease. Furthermore, we are gathering scientific data to prepare a clinical trial on treating muscle wasting in kidney failure. This research is supported by collaborating partners and national experts in the fields of muscle cultures and physiology, steroid hormone metabolism, drug development and chronic kidney disease. Hence, our own efforts and collaborations will inform plans to replace and reduce animal studies at the earliest opportunity.

Finally, we regularly review the relevant literature, attend professional conferences and participate in animal research training opportunities to attain continuous professional development on model-specific advances and animal husbandry in general. Continuous professional development activities also include engagement with resources offered by NC3Rs, such as subscription to the newsletter and attending local 3Rs events.

18. Modulating angiogenesis for 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

Angiogenesis, Cancer, Endothelium, Cell Adhesion, Microbiota

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The purpose of this project is to understand how we can modulate angiogenesis (blood vessel formation) for life-long health and to alter the course of disease.

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

Why is it important to undertake this work?

Irregular or un-balanced blood vessel formation (angiogenesis) contributes to a decline in health as we age and is a contributing factor in how large number of human diseases develop. The work performed on this project will provide novel insight into how angiogenesis is regulated so that we can help maintain normal angiogenesis as we age. This work will also advance our understanding of angiogenesis so that we can develop new evidence-based interventions to either stimulate angiogenesis in diseases where

there is not enough (e.g. cardiovascular disease) or to inhibit angiogenesis in diseases where there is too much (e.g. cancer).

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 angiogenic pathways integrate to regulate the process. This will include new information on specific molecules known to play a role in angiogenesis, and new information on how host-microbe interactions influence angiogenesis.
- Communicated our findings to scientist (e.g. by publishing our findings in peer-reviewed journals and presenting at scientific conferences) and to the general public (e.g. by engaging with social media platforms and by presenting to "lay" audiences).
- Begun the development of a microbiota based therapy that can be used to modulate angiogenesis.

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

Because blood vessels are essential to many physiological/healthy and pathological/disease processes, this research will provide insights into fundamental and translational mechanisms that underpin angiogenesis, and will thus benefit a wide range of individuals in both pure and applied contexts.

1. Ultimately, **people** wanting a therapy for maintaining a healthy vascular system throughout life, or patients needing to re-balance blood vessel formation as a disease treatment [>10 years].

2. **Biomedical researchers**. We believe that the mechanisms studied in this work are relevant to many human conditions, including age-related macular degeneration, arthritis, coronary artery disease, stroke and cancer. We expect that an increased understanding of the mechanisms that underlie normal and abnormal angiogenesis will facilitate progress in those areas. Moreover, we will be breeding new genetically modified mouse systems that will be useful to the research communities studying these diseases and we will make them widely available [during the lifetime of the project].

3. **Pharmaceutical companies/clinicians**. Angiogenic modulators have been developed and tested in clinical/pre-clinical settings. Their success, however, has been limited. The reasons for the failures are not fully understood. We anticipate that this project will uncover mechanistic interactions that help explain the, to date, disappointing clinical findings with the existing modulators. The findings from our studies may re-direct the use of current drugs and/or spur the development of new therapies (for example, we are trying to develop microbiota-based therapies geared toward re-balancing disrupted blood vessel formation. We expect the data generated from this study will inform future clinical trials and will impact on the design and implementation of future drugs and treatment regimens [beyond the timeframe of this project, >5 years].

4. **Staff associated with this project**. Training of staff working on this project will increase capacity in the fields of cell adhesion, microbiota, and angiogenesis, helping to create the next generation of scientists studying these processes [within the lifetime of the project].

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

The key outputs of the project will be communicated by:

- Publication in open-access scientific journals. We will seek to upload all material via preprint servers (e.g. bioRxiv; we have done this recently with all manuscripts) so as to provide project findings as quickly as possible to the wider research community. These publications will include both positive and negative findings.

- Meetings with current collaborators to discuss findings and directions. We also anticipate the work will attract new collaborations.

- Engagement with stakeholders (e.g. academics and the general public). This will be through our Institute Press Office, who will manage communication of the project outputs with the media in an appropriate and clear manner.

- Dissemination at national and international conferences.

- Further public engagement. We have extensive experience of engaging with the public about our science, and working in collaboration with our Institute communications team. We will establish project specific webpages on our lab websites, use social media (Facebook and Twitter), make public presentations via talks at science festivals and other outside events (e.g. Pint of Science), press releases, and blogs, throughout the project to communicate our research to the wider public, and also interact with broadcast media (press, radio, tv), via the science media center, and also partake in science programmes as appropriate.

Species and numbers of animals expected to be used

- Mice: 17450

Predicted harms

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

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

The mouse is an appropriate and extensively validated model for studying angiogenesis, both developmentally and pathologically. It is also a species in which reliable genetic engineering technology, microbiota manipulations, and germ free experiments may be performed. We have also engineered mice which are genetically altered in their ability to express proteins we believe play an essential role in angiogenesis (via tissue-specific inducible deletion); additional models will be generated in this project.

We will use embryos (up to embryonic day 10) to study early brain angiogenesis, as the central nervous system vascularises from embryonic day 7.5 up to birth. For these studies, procedures (e.g. substance administration) are performed on pregnant mothers.

We will use neonates to study postnatal angiogenesis in the brain as this organ continues to vascularise throughout early life.

We will use neonates to study retinal angiogenesis, as the mouse retina vascularises between postnatal days 1 and ~28.

We will use juvenile mice to study mammary gland angiogenesis as the mammary gland develops at the onset of puberty.

We will use adult mice for breeding, and for performing tumour studies.

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

Most animals used in this project will undergo one (or more) of the following procedures:

- **Tissue sampling** (to perform routine genotyping of progeny); typically **once only shortly after weaning**. This will involve around 80% of animals. Approximately 20% of animals are from wild- type breeding pairs that do not require genotyping.

- Substance administration via **oral gavage** (to induce alterations of the gut microbiota or in gene expression).
  - Neonates, typically 6-18 days, typically every other day. These animals are unlikely to undergo any other procedure.
  - Adults, typically 21-56 days, typically every other day. These animals are likely to also undergo orthotopic surgical implantation of tumour cells.

- Substance administration via **injection** (multiple routes - to induce alterations in gene expression, or to alter tumour progression).
  - Neonates, typically 6-18 days, typically every other day. These animals are unlikely to undergo any other procedure.
  - Adults, typically 21-56 days, typically every other day. These animals are likely to also undergo orthotopic surgical implantation of tumour cells .

- Orthotopic **surgical implantation** of tumour cells; typically once only.
- The remaining animals in this project will be used for **breeding**. Typically these animals are kept alive until they reach the end of their economic breeding life (6 months).

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

Potential adverse effects:

- **Pain** associated with tissue sampling (transient; no mitigation required).
- **Pain** associated with substance administration by injection or oral gavage (transient; no mitigation required).
- **Pain** associated with surgery (vasectomy, orthotopic implantation of tumour cells, mammary fat pad clearing and transplantation - several hours; mitigated against by administration of analgesia pre and post surgery).

- Discomfort associated with **tumour** growth (days; mitigated against by Schedule 1 killing at first signs of poor health condition - see Table 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)?

Mild **pain** associated with tissue sampling; 80%.
Mild **pain** associated with substance administration by injection or oral gavage; 80%.
Moderate **pain** associated with surgery; 35%.
Mild to moderate discomfort associated with **tumour** growth; 35%.

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 use of animals for this project is essential; we need to determine how blood vessels form in whole tissues which contain multiple cell types that must interact with one another and without damaging the tissue in which the vessels are forming. Moreover, the reciprocal interactions occurring between organismal level systems (such as the microbiota and/or the immune system) and the local tissue microenvironment are complex. These interactions cannot currently be readily replicated outside the endogenous physiological environment

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

- In vitro culture of endothelial cells.
- Ex vivo culture of aortic rings.
- Organs-on-chip models.
- In silico modelling.

Why were they not suitable?

We do use in vitro and ex vivo assays of angiogenesis where possible (see project overview). Moreover, we frequently immortalise endothelial cells (the major cells that make up blood vessels), thereby reducing our need to continually isolate primary cells which have a limited life span in culture. However, these assays, as they stand, cannot adequately model the complete array of cellular and tissue interactions that are important during both normal and abnormal angiogenesis; angiogenesis involves many different

cells types (e.g. pericytes, leukocytes, fibroblasts, etc.) including endothelial cells. In addition, removing cells from their natural environment rapidly induces changes in their functions. Finally, growth factor gradients and tissue oxygenation patterns that depend on blood flow cannot be reliable reproduced in the in vitro or ex vivo systems. Both parameters dramatically influence angiogenesis. Organs-on-a-chip are being developed, but they do not yet reliably model multiple interactions between very complex systems such as the microbiota, the immune system, and local tissue microenvironments. Thus, while we will continue to make the maximum possible use of cells/tissue from animals that do not undergo regulated procedures, it is currently not possible to perform the experiments described in this application without performing procedures on living animals.

Finally we are beginning to form collaborations that will allow us to do some in silico work, but these collaborations are still young, and the methods 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?

Many of the procedures and protocols described in this project have been developed and extensively used during the previous PPL and have produced scientifically robust data that has been published in peer reviewed journals. The total number of animals to be used in this project is therefore primarily based on the use of established protocols. These totals include control groups (e.g. mock or placebo treated) for each experiment which are of the same size containing animals of the same age and sex distribution as the experimental groups, in order to increase confidence in the validity of the data and that any significant findings are attributable to the intervention. The protocols also include replication which is necessary to ensure that the results obtained are reliable and repeatable and not due to operator bias or other extenuating circumstances. Experiments are routinely planned to maximise efficient animal use by, for example, sharing tissues, particularly from non-treated control animals, with other investigators and by sharing data with other investigators to help inform their own studies. We also routinely bank large numbers of tissue samples from all animals for future use in the same or subsequent studies. For those protocols new to this proposal we have consulted with the in-house establishment statistician together with reviewing published studies that use the same or similar protocols to determine the minimum number of animals to obtain scientifically robust findings.

With regards breeding, and whether or not to set up and breed a colony in-house we first evaluate the scientific need and whether the number of animals required and the anticipated timescales justify breeding in-house. We also consider whether the conditions animals have experienced prior to starting the experiment may influence the science and must be standardised. For example, given variabilities in microbiota across different

facilities, and how this can contribute to different experimental outcomes, it is often more scientifically sound to breed in-house than to buy animals (e.g. with wild-type colonies).

Regarding colony maintenance, we perform breeding calculations (frequently in consultation with establishment staff who keep records on colony breeding efficiency) before we plan our experiments and only produce the numbers of animals that we need which is in line with Home Office guidance (Assessment Framework for the Efficient breeding of GA Animals). See below.

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 in each protocol has been projected based on the following:

- Previous experience with a specific protocol (we are experienced in the use of all protocols).
- Experimental design planning (and post analyses, see below) in consultation with in-house statisticians, to ensure at the out set of the project that protocols are sufficiently powered.
- Use of the NC3R's Experimental Design Assistant, particularly where novel parameters are being assayed.

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

- **Efficient breeding** of GA lines – GA lines are routinely maintained by keeping ~2-3 breeding pairs per line over the course of the year. This ensures sufficient stock for maintaining the line (we replace pairs when they reach the end of their economic breeding life, typically 6 months) and generally produces enough animals for experimental procedures. Where neonates are required for substance administration to induce changes in gene expression or microbiota composition, dedicated pairs are established solely for this specific purpose and the pairs are culled as soon as experimental needs have been met. Mice under sporadic use are maintained at lower levels and lines are frozen down whenever practicable. We also seek colony management guidance from technical staff within our establishment and from the NC3R's guidance page on breeding and colony management. With these inputs we routinely self-assess the breeding efficiency of our colonies (GA and WT) - this ensures regular review of colony performance so as not to exceed requirements for prioritised experiments. Where possible I maintain collaborations with other on- site licence holders to minimise over-breeding and duplication of experiments.

- Specific mitigations to minimise animal numbers:
    - **Tumour studies** – Tumour lines expressing luciferase or other biomarkers are used wherever possible (in transplantable tumour models) to determine tumour growth and/or metastases longitudinally via in vivo optical imaging. This is a non-invasive technique providing consistent data leading to fewer required animals.
    - **Histological studies** – For qualitative experiments, the amount of material required will be the minimum necessary to provide an adequate description (e.g. of vascular anatomy).Wherever possible, we share tissues with other licence holders to maximise the use of any one animal.

- **Substance administration** – Littermate controls and the use of contra-lateral mammary glands as internal controls provides for accurate assessment of treatment effects. For i.v.,i.p. and subcut. routes of injection, advice from the experienced animal care personnel within our establishment will help maximise consistent substance delivery, minimising the number of animals required.

- Ensuring our data is as robust as possible:
  - **Statistical analyses**. Where are are measuring new/unique parameters, we will employ **pilot studies** using a minimum number of animals, and the smallest amount of suffering, to achieve an experimental objective. These studies will involve two groups only (control and treatment). Effects and standard deviations within groups will then measured and statistical analyses performed, with assistance of in-house statisticians. Power calculations will then enable us to estimate the number of animals required to generate statistically-significant results, and these predictions will guide the decision making process. We also perform meta-analysis on data from pooled experiments to reveal less pronounced effects, thereby reducing overall animal use.
  - **Randomisation**. Wherever possible animals will be completely randomised into experimental groups (e.g. using the =Rand() function in Excel). This will prevent the bias of a covariant on the outcome of the treatment.
  - **Blinding**. Blinding (colour or numerical coding) will occur at multiple stages
    - During treatment. Those administering treatments or surgical interventions will be blinded to the treatments.
    - During result assessment. Those taking longitudinal measurements will be blinded to treatments.
    - During data acquisition and analysis. Those acquiring data or analysing data will know animal groupings, but not treatments.

  - **Methods of analysis**. For data analysis the type of statistical tests to be used depends on the number of variables/groups to be compared, the distribution of the data, and the outcome of consultations with the institute statisticians. We will also employ the expertise of in-house statisticians to perform meta-analysis on data from pooled experiments to reveal less pronounced effects, thereby reducing overall animal use. Conducting and recording of experimental data will follow ARRIVE guidelines.

- **Experimental repeats**. Most experiments will need to be performed on at least three occasions (N=3) to ensure reproducibility.

Refinement

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

Which animal models and methods 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 of choice:

- The mouse brain, retina, and mammary gland are extremely sensitive vascular systems providing valuable information even in heterozygous animals. In the methods we are employing, their use is largely restricted to animals that have undergone substance administration (to induce changes in gene expression or microbiota composition) via minimally invasive procedures (e.g. oral gavage or injections). Routes of administration, volumes, and frequencies are designed such that they elicit no more than transient discomfort and produce no lasting harm.

- Most of the GA mice used in this project have been generated to produce tissue specific timed gene activation or inactivation in expression of genes we believe to play essential roles in angiogenesis. None are expected to produce harmful phenotypes. These types of models provide the best possible data with greatest detail of mechanistic information, with minimal invasiveness. However, where new models are being created, it is not possible to fully predict the nature or severity of any potential defect. Therefore, for all types of mice there will be careful monitoring for possible adverse effects. Animals exhibiting any unexpected harmful phenotypes will be killed, or in the case of individual animals of particular scientific interest, advice will be sought from the local Home Office Inspector.

Specific methods -

- Surgery: Recovery surgery will be done aseptically according to Home Office guidelines (Minimum Standards for Aseptic Surgery and LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery).

- Analgesia: For pain relief, peri and post operative analgesia will be given and maintained after surgery for as long as is necessary to alleviate pain. Non-stimulus evoked methods (e.g. gait analysis and grimace scales) will be used to quantify “pain-like” behaviour.

- Microorganisms: these will be administered at doses known to induce an appropriate change in microbiota composition. Where the outcome of colonisation is unknown, dosage will be carefully titrated using small-scale trial studies to minimise any adverse unknown effect.

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

Mice are the lowest form of sentient species that can be used for the studies we propose where multiple systems are interacting to regulate angiogenesis within a tissue without disrupting the overall histoarchitecture of the tissue. Because mice and humans are mammals, their mechanisms of angiogenesis, and the systems that come into play, share many similarities. Moreover, because mice are commonly used by other angiogenesis and microbiota researchers, there are many useful materials that have been developed by other scientists that are available for our work.

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

We have specifically generated GA mouse strains that affect the endothelial targets we are suggesting as playing essential roles in both normal and abnormal angiogenesis so that we may test our hypothesis.

We use routine and well-tested procedures for studying angiogenesis that provide the most robust data. The procedures we employ have been refined over the years to cause minimal discomfort/adverse effects to the animals. For example:

- We use anaesthetics and analgesics appropriate to each technique.
- We routinely monitor animals for health status, particularly during tumour studies and after surgical manipulation (this includes the use of gait analysis and the mouse grimace scale to assess pain -doi:10.1038/nmeth.1455)
- We try to avoid stress by training animals to cooperate with our procedures.
- The staff in our establishment provide animals with appropriate housing that allows the expression of nesting opportunities.

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

As mentioned above, we will subscribe to the NC3Rs e-newsletter and regularly visit the NC3R website so that we may stay abreast of current best practice in the 3Rs. We also actively monitor the published literature in our field(s) for updated/refined techniques being used by other researchers that may be employed by us. Where possible, we will visit other labs to refine our methods. For example, we collaborate with other groups to ensure your own group's skills are kept up to date.

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

- We will regularly engage with establishment technicians/staff to review current approaches and discuss whether there are any new 3Rs opportunities.
- We will subscribe to the NC3Rs e-newsletter and regularly visit the NC3R website so that we may stay abreast of current best practice in the 3Rs.
- Will will discuss 3R updates at our regular colony management meetings - this will ensure that updated 3Rs best practice is employed as soon as possible.

19. Nanomedicine platforms for pancreatic cancer therapy

Project duration

5 years 0 months

Project purpose

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

Key words

Pancreatic cancer, Drug delivery, Nanomedicine, Theranostic, Nanotechnology

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?

Pancreatic cancer therapies have not had a major impact on patient outcomes over the past 40 years, nanotechnology is one such platform which has shown great promise in this area. The purpose of this project licence is to evaluate various nanomedicines and nanotechnologies in order to determine whether they are safe to use, better than existing chemotherapy and ultimately whether they should progress to clinical trial.

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

Why is it important to undertake this work?

Pancreatic cancer is one of the worst cancers in the western world, currently once diagnosed patients only have a 6% chance of surviving up to 5 years. Pharmaceutical companies are not willing to invest in new technologies for pancreatic cancer therapy without a very solid in vivo case showing that they are more beneficial than whats already

available. This is because pancreatic cancer is so difficult to treat and therefore a lot of new candidates fail in trial. Unfortunately, there is still no adequate non-animal models which can be used to predict in vivo fate and so we must carry out these preliminary trials on mice.

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

The data acquired from the studies under this project licence, will provide new information as to the feasibility of the novel nanotechnology platforms being used clinically. The data will be collated, analysed and disseminated where possible (unless under intellectual property protection) through academic publication and talks at conference. In addition, dissemination will be used in lay form to engage with clinicians, patients and local support groups to inform them on the progress within the field. Specifically, the outputs will be:

Nanocarriers / drugs / formulations will be tested in order to determine their suitability for clinical use based on a series of testing methods.

It is estimated that 3-5 novel formulations will be tested and reported over the 5-year project licence duration.

Evaluation of pharmacokinetic, pharmacodynamics, safety and efficacy, and anti-cancer activity of the novel formulations identified and reported.

Level of tumour retardation compared with clinically used chemotherapy will be evaluated as the measure of success.

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

The output of this project will inform as to whether it is worth taking the nanomedicines forward to larger scale or clinical trial. This is a vital piece of information and is required to remove any formulations or iterations of the technology which are not suitable especially due to toxicity problems or adverse events which were not picked up in the in vitro studies.

Long term benefits are the healthcare providers as more effective therapies lead to less cancer recurrence and patient bed-time, so economically these could be favourable. But ultimately all the work in our group focusses on patients as an end point, and we aim to provide better therapies for patients diagnosed with pancreatic cancer.

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

Science is now global with collaborations across continents easier than ever. I work with the best clinicians and researchers within this field to help drive forward the technologies we develop.

Dissemination will happen through the usual route of conference presentations and academic publications, but also in talks with clinicians, patients and support groups. My group have strong links with pancreatic cancer charities, and do a lot of public engagement activities surrounding this, including our recently funded Royal Society of Chemistry outreach grant called Quilting for Cancer, where scientists across the UK

design quilt patterns based on their research which volunteers form into quilts. These are then donated to pancreatic cancer patients who are often cold during their chemotherapy.

Such a project would include the concepts or data collected from the projects within this licence.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

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

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

We have chosen the species least likely to suffer distress without compromising the likelihood of studies working. We will use special mice with a defective immune system to prevent them having a reaction against the tumour cells. This is the most commonly used species for this type of test.

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

Animals will have small volumes of cancer cells injected to allow a tumour to grow inside them. This may cause modest discomfort. The size of the tumour will be carefully monitored and animals will be humanely killed before it becomes large enough to cause distress. Drugs may be administered to the animals by injection. The process of administration of the drug is only expected to cause very mild discomfort to the animals. Additionally some animals may be anaesthetised and their tumours irradiated with a laser light, which will help initiate the therapy. The study duration is 4 weeks of treatment, with 4 weeks of monitoring after the treatment has ceased.

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

The adverse effects of the nanomedicines themselves depend on the type of formulation we test and which drug they incorporate. Sometimes we are testing drugs that have been used before and we know what adverse effects to expect. However, many of the drugs are new and although they are designed to specifically target the cancer cells, they may have unexpected effects. This makes it hard to know what adverse effects might occur.

To minimise the adverse effects on the animals, we will first test the drug on a small number of animals to identify a dose of drug that has only moderate adverse effects. All further experiments will use lower doses, so we anticipate that the majority of animals will only experience mild severity events in some studies, we may take blood samples (again with a narrow needle) and only small volumes of blood will be collected as infrequently as possible. In some studies imaging of the animals will be carried out, the animals will be injected IP with appropriate dyes, anaesthetised and placed inside an imaging cabinet.

In some studies we will also use laser irradiation to the localised tumour site in order to initiate drug release from formulation. In this instance, the mice may develop scabbing around the area of laser irradiation.

At the end of the experiment, 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)?

Moderate for all studies

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

- Killed

Replacement

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

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

We wish to find new nanomedicine formulations to treat pancreatic cancer. We are testing nanomedicines which specifically target cancer cells incorporating drugs that have been used clinically as well as drugs that are completely new. We need to gather sufficient information to convince the medical and pharmaceutical world whether our formulations are likely to work in patients – it is not reasonable to give a patient who is already quite ill a new drug unless there is a reasonable likelihood that the drug will do more good than harm. To gather this information, we need to test the drugs using a method as close to real patients as possible. Although we can (and will) test the drugs in the laboratory, these types of studies are not sufficiently complex to model what happens in real patients. Animals are not a perfect model, but they are the closest model we have. Using computer simulations can help, but simulations are limited to testing things we already know about. Our formulations are completely new, or they are established drugs being used in a different way, so we don’t know what will happen. We can’t make a computer model of something we don’t know about.

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

Ex vivo systems such a spheriods or tumour development through microfluidics is reported in the literature.

Why were they not suitable?

The technology is not fully developed for either to give the level of detail which is acquired using the in vivo models. Unfortunately, currently there is no suitable alternative and thus

we must use animals. We do as a group constantly scour the literature for alternatives and when a reliable alternative is available we will use this in place of in vivo trials.

Reduction

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

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

Based on usage in previous licence and estimated projects under this 5 year licence

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

We will design our experiments to minimise the numbers of animals used.

Firstly, all the nanomedicines which are tested in animals will be tested in several different types of experiment in the laboratory to confirm they have the desired anti-cancer activity. We will also try to mimic in the laboratory the conditions in the body. If the drugs do not work in these studies, we will not test them animals. This “triage” process will minimise unnecessary testing.

Secondly, we will conduct studies in the laboratory that form a “bridge” between the laboratory studies and the animal studies. In these we will try to estimate how often the drug needs to be given to the animals. When we move to testing the drug in animals, we will first conduct experiments with a relatively small number of animals to establish the correct dose of drug and the frequency at which is administered.

Both these strategies will minimise the chance of an experiment having to be repeated because it was incorrectly designed. We will also use statistical principles to estimate the minimum numbers of animals we need to use to measure the effect of the drug.

Experimental design will be designed and reviewed in consultation with the named Biostatistician and through Dr Michael Festing’s website www.3Rs-reduction.co.uk . Pilot studies may be performed to assess experimental viability and use will be made of historical and/or published data to appropriately power the study. Prior to each experiment, a literature search will be performed to ensure that the planned work is not duplicating that performed previously by others. The result of the study will be disseminated following the ARRIVE guidelines, to ensure that all outcomes of the work.

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

We plan to purchase animals on an “as-needs” basis from commercial vendors. Inbred strains will be used to reduce variability. We do not plan to maintain a colony of nude mice because the frequency of experimentation would lead to surplus 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.

These studies will be performed with animals of the lowest neurophysiological sensitivity possible. Immunocompromised animals are required, because the animals will usually receive a human xenograft. Nude mice are commercially available and routinely used for these studies, and will be used here. Animals will be maintained in an environment that supports their normal behaviour.

The nanomedicines will be administered by trained personnel through a narrow needle to minimise pain. The animals will also be closely monitored after they have received the formulations and if the adverse effects appear to reach moderate severity, the animals will be humanely killed. In this way, we expect very few animals to experience more than relatively modest adverse effects.

All of our studies have been reviewed to consider how to minimise costs to the animals. Animals will be maintained in an environment that supports their normal behaviour We have considered any likely adverse effects of the procedures we will use, and we will regularly monitor the animals to make sure that the animal are not distressed. We may use optical imaging to help us monitor growth of tumours. Staff will be trained to recognise signs of distress and how to deal with this situation.

Where new nanomedicines are used, the effects of new formulations may not be predictable. Therefore, a low dose, as informed by the in vitro work, will be used and the dose escalated until risk of exceeding moderate severity effects is encountered or when successful outcome is reached.

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

The less sentient option would be to work on zebra fish models. However, these are not yet developed enough to provide use the information we gain from mouse models.

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

To minimise suffering, procedures have been designed to evaluate the animals closely during these studies. These include regular monitoring, use of analgesia or other medication as required and euthanasia if necessary. Subsequently, the dose, route or schedule of drug administration may be altered to reduce harmful effects.

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

Dr Michael Festing's website www.3Rs-Reduction.co.uk Experimental Design system implemented by NC3Rs

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

As a group we are constantly assessing the literature for new pancreatic cancer models, discussing with clinicians and collaborators on their effectiveness. Additionally, we stay fully engaged with the work within the NC3Rs research council and output from their work. When a suitable alternative model is available we will switch to using it.

20. Understanding the mechanisms governing ovarian ageing and infertility

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

Reproduction, Ovary, Ageing, Infertility

Animal types	Life stages
Mice	adult, embryo, juvenile, 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 determine the mechanisms governing normal ovarian ageing and how this is dysregulated in disorders resulting in infertility and premature ovarian 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?

Ovarian ageing is a naturally occurring process that results in changes in the hormones produced and ultimately in the cessation of ovarian function. As well as causing infertility, ovarian ageing also increases the risk of developing several life-changing diseases, e.g., brittle bone disease, heart disease and impaired cognitive functions. This not only impacts on quality of life, but also results in a significant cost burden to the patient and Health Services. Despite this, our current understanding of how the ovary ages and what

determines the lifespan of the ovary remains limited. Surprisingly, current treatment options for declining in ovarian function are limited, with assisted reproductive technologies including IVF, the preferred treatment option for infertility, which has limited success rate with increasing age. Moreover, hormone Replacement therapy is the only treatment option for delaying of secondary associated health issues, but this can only be taken for a limited time and cannot be taken by all women. This project aims to use mouse models to understand the physiology and molecular mechanisms underpinning normal ovarian ageing and how these are modulated in disorders that result in premature ovarian ageing/infertility. This is essential to highlight novel therapies for preventing/delaying ovarian ageing, to improve quality of life.

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

On completion of this project, we will have a greater understanding of how changes in the hormones that occur with age impact ovarian function and contribute to ovarian ageing. Expected outputs will include primary research publications, dissemination of research via conference communications and through public engagement and outreach activities including talks with public and schools.

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

The primary and immediate benefit of this work will be to advance the fundamental scientific knowledge about how the ovary ages in physiological and pathophysiological conditions. We aim to publish our findings in scientific journals, with the study findings likely to be of interest to physiologists, endocrinologists, ageing biologists and cell biologists, with an interest in the mechanics of reproductive physiology.

A medium-long term potential secondary benefit relates to possible clinical application of our findings to infertility and ovarian ageing in humans. Understanding the changes that occur to the ovary as it ages may highlight novel treatment avenues for infertility, and for potential treatment of the secondary health problems associated with menopause.

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

To maximise the outputs of this work, the work will be disseminated via conference presentations and publication of the study findings to the wider scientific community. We have collaborations with researchers from other institutions, both nationally and internationally, in fields outside of ovarian ageing, which will ensure that this work reaches other fields as well as our own. If the findings of this study are of therapeutic potential, collaborations with industry will be sought to explore translational potential.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

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

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

Rodents (mice) are the lowest vertebrate group which will allow reliable reproductive physiology studies, in which the reproduction control systems have been well characterised. They are excellent for monitoring ovarian function via oestrous staging and superovulation techniques (hormonal stimulation of the ovaries to produce more than the normal amount of eggs).

Since we are examining the changes in ovarian function across lifespan, neonatal and juvenile, adults and aged animals are required. As the ovarian architecture is completed early in postnatal life, studying of neonatal ovarian composition is required.

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

Typically, genetically modified animals will be bred, genotyping will be conducted using tissue biopsy collection. Females may be staged for oestrous cycle and mice killed at key time points during their reproductive lifespan, using Schedule 1 methods, or exsanguination with killing confirmed via Schedule 1 methods.

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

We expect minimal impact and adverse effects to the animals during the project.

There is a low risk (<1%) of blood clots or leakage of fluid from blood vessels following intravenous injection or perforation of an internal organ following intraperitoneal injection. Any animals displaying signs of adverse effects (as judged from food and water intake, body weight, general and coat appearance, gait or behaviour) will be humanely killed by a Schedule 1 method. Animals will be monitored daily for complications and advice sought from the NACWO/NVS.

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

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

Expected severities are mild for all regulated procedures.

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 programme of study will use in vitro and ex vivo techniques and animal procedures. The in vitro and ex vivo work will examine biological activity in selected cell lines and tissue to elucidate signal transduction pathways and gene expression using molecular biological techniques. However, animal work is essential to understand as ovarian function is regulated by integrated physiological and endocrine systems, making it impossible to study infertility and ovarian ageing in cultured cells alone.

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

Non-animals alternatives considered were ovarian cell lines.

Why were they not suitable?

Ovarian cell lines often lack key receptors and the ability to produce hormones that are essential ovarian factors. Ovarian cell lines are currently very limited and do not provide the ability to assess aspects of communication between the germ cells (oocytes) and the surrounding somatic cell tissue. Moreover, assessment of infertility and ageing is difficult in a cell-line based experimental set-up. Thus, the use both primary ovarian tissue and assessment of whole animal physiology is required.

Reduction

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

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

We have drawn upon our own experience and published studies to ensure we use the minimum number of animals required. We will use statistical power analysis where appropriate before beginning experiments to ensure that we only use the minimum number of animals required to produce valid statistical comparisons between the groups.

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

The design of individual experiments will generally involve factorial designs, to maximise the information gathered using the minimum number of animals. Most measures taken will be quantitative, in which case sample sizes will be set using a power analysis, using a significance level of 5% and a power level of 80%. This will generally result in group sizes of ~8 per treatment group. For design of complex experiments, advice of a statistician will be sought.

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 factorial designs will maximise the information gathered using the minimum number of animals per experiment. We will also use data generated from in vitro cell lines to inform and reduce the number of animals used in future experiments. We will utilise full post-mortem tissue profiling to maximise the utilisation of tissue, and ensure a small number of animals are made use of as much as possible.

Refinement

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

Which animal models and methods 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 (including mice) are the lowest vertebrate group which will allow reliable reproductive physiology, in which the reproduction control systems have been well characterised and elucidated. They are excellent for monitoring the ovarian function and fertility. Moreover, mice are most commonly used for genetic manipulation/alteration, making these an ideal model for studying genetic modifications that result in infertility and premature ovarian ageing.

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

Rodents are the lowest vertebrate group which have been highly characterised and extensively used for studying reproductive physiology. This project will heavily utilise 3D ovarian organoid cultures to study aspects of ovarian ageing and infertility. However, assessment of ovarian function, cyclicity and fertility can not be achieved in vitro, nor using less sentient animals.

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

Animals will be monitored throughout all experiments to minimise pain or discomfort. Any animals deemed to be suffering will be euthanised using a Schedule 1 method.

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

We will follow the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) guidelines, and ensure we keep up to date on current and new practices in the scientific literature to ensure our 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 stay informed on new advances in the 3Rs through the NC3Rs newsletter and through regularly checking the 3Rs website.

21. Models of tissue injury, regenerative repair, and reconstruction in the limb and appendages

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Injury, Wound healing, cell therapy, biomaterials, tissue engineering

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

We aim to study how animals that have an enhanced healing ability, respond to injury to their limbs, digits and ears. This will allow us to understand how these tissues regenerate, and where possible capture these mechanisms to use in regenerative biomaterials to repair wounds and create new tissue.

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

Why is it important to undertake this work?

If we can understand how certain mammals are able to regenerate and heal better, and understand how new tissue is formed, after injuries to their limbs, we can improve on our

interventions such as surgery, and treatments to restore form and function after trauma, disease, or cancer excision. As surgeons, we see these demands on the NHS, and perform repairs and reconstructions on a day to day basis.

Improved healing or engineered tissues that are biocompatible, to replace missing tissues would represents a major shift in surgical care.

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

The hope of this project is to understand how tissues can be regenerated or replaced to develop new therapies towards the clinic. This may be by development of a new biomaterial therapy or engineered Replacement tissues for patients. We would seek to publish these innovations throughout the course of this project, like we have done in the past. There is much learning to be had from this work and as we have found in the past, when new discoveries are made, we can use the information to modify surgeries, generate high impact publications, use the data to apply for further grant funding, and develop new therapies and patents.

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

Patients who sustain traumatic injury or organ damage will be the beneficiaries of these outputs in the long term. All parts of the study are designed to have line of sight to patient benefit through our clinical group. This includes rapid translation of the science to change surgical practice (which we have previously demonstrated), but also development of new novel therapies, such as biomaterial and cellular therapies. Our work on engineering blood vessel networks, has the potential to allow for new tissue and possibly Replacement organs to be engineered that would have a significant impact on people’s lives, with conditions or injuries requiring tissue Replacement therapies. We would aim to have successful clinical impact within 10 years.

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

All projects are collaborations between scientists and clinicians, from the biomaterial, cell biology and translational science field. Knowledge will be disseminated through publication and international presentation at both clinical and scientific conferences. Positive results towards clinical benefit will be subject to further grant funding and opportunities to translate wherever possible, this may be in tandem with industry partners.

Species and numbers of animals expected to be used

- Mice: 3500
- Rats: 700

Predicted harms

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

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

Mice and rats are being used in the adult stage of their life stage. The justification is based on the fact that these are the lowest sentient mammals, that have anatomical structure and biological processes that are similar to man. Hence the study of injury and repair on these animals provides us with invaluable insight on how they can better heal. Our surgery and anaesthesia can be safely performed on animals of this age and size.

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

Our study will broadly cover three areas of wound healing interest.

How do animals that regenerate, heal wounds of the digit and ear?

Can we produce regenerative therapies from our understanding of this process?

Can we engineer vascularised tissue/organs that are stable and functional?

We will be breeding animals that have specific genetic changes that will allow us to examine particular areas of wound healing that may allow us to understand how to improve on the healing process. This would require animals that heal better or heal worse, or have genetic characteristics that enable us to understand and visualise the healing process better.

Testing of these animals to have the correct genes will occasionally require us to take a small blood sample from the tail or a small sample from the ear which may cause transient pain.

Some animals will undergo chemotherapy to clear their bone marrow and allow for bone marrow to be transplanted which will allow us label cells in their bone marrow or change the characteristics of their bone marrow cells which may alter how they heal. This would allow us to examine the importance of bone marrow in the healing process. The bone marrow in these animals is always restored to bring the animals back to health.

Within each study, animals will be subject to injuries that are simplified versions of injuries seen in the clinical setting, and then observed for how they heal. This will involve either a cut across a finger or ear where there is no loss of tissue, or excision of tissue across the finger or ear, where there is loss of tissue. Injuries will be studied in animals that can regenerate, and by understanding how they heal better, we will try to harness the biology and apply them to biomaterials as a potential therapy. These injuries are small (several millimetres) and cause minimal pain, distress, and disturbance to the animals. As such these small injuries can be performed on the left and right paws or left and right ears without much disturbance, hence allowing for comparisons of how treatments work without using more animals and without more suffering.

In addition, we will be using our understanding of healing to generate new tissue. This will involve combining surgery, biomaterials, and cell therapies to enhance tissue growth and development into Replacement tissue that may be used to transplant to other animals as a Replacement for damaged tissues.

These experiments will be conducted to observe the full healing process, which is typically 3 months, and the number of procedures will be kept to the minimum required for us to obtain a meaningful understanding of the biology (usually 1 but up to a maximum of 2 per animal). The information collected has to be of sufficient quality so that we can reliably develop therapies for patients

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

We aim to use only animals that are healthy in our study but in generating animals used for tracing of particular cells of the bone marrow, this necessitates chemotherapy and bone marrow transplantation. This makes animals susceptible to infection and we try to reduce the chances of this by acidified water, antibiotics and clean caging.

For these studies we will be performing very simple injuries on the digits or ears. These simple injuries can cause pain, bleeding and impact on mobility, but all of this is transient and allow for quick recovery. Having developed these procedures, it is rare to see anything more than transient discomfort in the animals or abnormal behaviour.

In some animals we will be implanting biomaterials that may have a biological effect. They are all tested to be biocompatible and should allow for better healing. However, they may rarely cause tissue reactions that are usually transient. We will provide pain relief to limit the symptoms from this. We also have vascular tissue engineering model that hopes to grow new tissue and organs. Rarely the cells may grow into benign tumours. If this occurs and limits the function of the animals, the experiments will cease.

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

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

90% of the mice bred on the licence will be expected to experience sub threshold harms with 10% possibly experiencing mild harms for differences in their genetic make up.

100% of mice undergoing bone marrow transplantation will be expected to experience no more than moderate harms.

100% of mice undergoing surgical procedures which include; 1. injury and repair, or 2. Vascular tissue engineering or 3. Vascular tissue transplantation will be expected to experience no more than moderate harms

100% of rats undergoing surgical procedures which include; 1. injury and repair, or 2. Vascular tissue engineering or 3. Vascular tissue transplantation will be expected to experience no more than moderate harms

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

- Killed

Replacement

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

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

After tissue injury there are changes in blood flow, inflammation, the formation of blood vessels and tissue remodelling, including interactions between numerous cell and tissue types. No in vitro cell culture can study these interactions in concert.

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

We have tried to mathematically model these changes to replace the use of experimental animals and are exploring ex vivo models using whole limb perfusion, and organs on a chip technology that will run in parallel to these studies. If specific questions can be answered and validated on these systems the use of animals can be reduced and replaced.

Why were they not suitable?

Mathematical models are not able to model the complex interactions of systemic inflammation in a spatial temporal fashion. The information acquired in vitro and in silico is insufficient to allow progression to trial in human patients.

Reduction

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

These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

We have estimated the numbers of animals used based on our current throughput of animal experiments, what is technically feasible to perform, and the planned scientific questions we are planning to answer over the next 5 years. We have considerable experience in planning the right numbers of animals for the research questions in order to maximise use, and minimise waste. This is also considered in the context of our current group make up and the projects which are funded. The minimal number of animals are used to allow us to answer the posed scientific question.

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

We are constantly evaluating ways to obtain the most information from the smallest number of animals, and this includes using more sensitive methods of quantification, using numerous modalities of analysis on one sample and ensuring the experiments are well-

designed to minimise animal waste. All animal procedures are only conducted once protocols have been discussed with in-house animal facility expertise, including the vet, and signed off. This is supported by pilot experiments to ensure feasibility prior to embarking on bigger studies. We are always looking for other models that can potentially provide us with the same information that does not harm animals including experiments that can gain the data direct from patients. Animal experiments are only considered when the information cannot be obtained from other publications or the clinical setting.

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 conduct experiments with the necessary pilot studies so we are in the optimal condition to proceed with larger numbers of animals. We are now also taking more of a systems biology, and non- destructive sample approach to allow us to generate far more information from each animal than previously possible. This is done with comparison to known datasets and in silico models which reduces animal numbers.

Refinement

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

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

We have developed the simplest injury and repair models that create the least disturbance and suffering on the animal which still provides us with useful information to take back into the clinic

Where abnormal behaviour is observed, this usually indicates that the animal is suffering and as such we limit any further involvement of the animals in the experiments. We will monitor for any suffering and where necessary intervene with pain relief, water and food. If this is insufficient, the use of animals in study will end and they will be humanely killed.

We will use models developed in the mouse and rat which are the lowest sentient animals for the purposes of mammalian study. Mice are used for their genetic tractability that allow us to examine the role of inflammation after injury, and rats are used because some regenerative biomaterials cannot be fabricated to a small enough size and resolution to be used in the mouse. The engineering of vascular tissues in the mouse is unique to our group and will be used in our studies to engineer tissue for Replacement tissues and organs.

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

Mice and rats are the least sentient animals that have relevant mammalian biology and also, they have anatomical structures that are similar in composition and configuration to man. As we are interested in how the body heals after injury, it is important that we study how these animals recover as you would expect humans to do the same.

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

All injury models are developed with clinical scenarios in mind and each surgical procedure has been refined to its simplest form.

All surgeries are designed to be quick, precise, and optimise workflow in theatres. Sterile equipment is used throughout. Complications are carefully monitored for and are fortunately rare. We continue to ensure all procedures lie well within the moderate severity range. The experimental models outlined are well validated, reproducible and consistent injuries, with the minimum amount of pain, suffering, distress and lasting harm. We have many years of experience in refining our techniques and all team members have skill sets attained through extensive training and/or clinical experience. For precision all procedures are performed under high magnification.

In addition to our surgical models, our use of bone marrow transplant models have undergone Refinement by moving away from harsh radiotherapy based bone marrow depletion, to using chemotherapy based depletion which results in far more reliable and less harmful bone marrow depletion. We monitor these animals carefully and provide acidified water, irradiated diet and prophylactic antibiotics to minimise chances of infection.

Suffering is minimised through careful monitoring and by use of analgesia, and good husbandry. We have reduced variability in the surgeries over the years that allows us to obtain meaningful, reproducible results that will continue to be refined. We also ensure careful training of all our staff to be mindful and skilful at surgical procedures and animal care.

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

We use guidance from PREPARE and ARRIVE to ensure the standards of our animal experimentation and reporting allow for the quality, reproducibility and translatability.

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

We regularly receive updates from our establishment about new and novel surrogates to animal experimentation. We regularly keep up to date with published activity and calls form the NC3RS and have applied for previous awards. In addition, we actively are trying to develop our own systems that do not use living animals, such as ex vivo perfusion and organs on a chip technology, or perform investigations whenever possible on patients themselves, and perform scientific outcome measures on tissue from patients via our biobank.

22. The development of rodent cancer models and their pre-clinical use as a platform for drug development and drug evaluation.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, Anti-tumour

Animal types	Life stages
Mice	adult
Rats	adult, juvenile

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project licence is to identify potential drugs / therapies that reduce, inhibit or prevent the growth of tumours leading to new and improved cancer 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 benefit of this project will ultimately be the introduction of new and improved

treatments for the management of cancer.

There were 9.6 million deaths from cancer worldwide in 2018. Lung, liver, stomach, and bowel are the most common causes of cancer death worldwide, accounting for more than four in ten of all cancer deaths. Lung, liver, stomach and bowel cancers have been the four most common causes of cancer death since 1975.

Based on our past record we would expect to deliver 10-15 potential new drugs into clinical development during the period of this licence. Whilst not all of these will be successful, as we move to more novel and sophisticated approaches to cancer management, it seems reasonable to predict that approximately 20% of the nominated compounds will reach people living with cancer and provide significant benefit to cancer sufferers. Since most of our approaches here are applicable across a broad range of tumour types it is likely that these could have wide utility. These may not completely cure cancer patients but it is expected that they will enable a significant number to live longer with improved quality of life following the diagnosis of this devastating disease.

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

The benefit of this project will ultimately be the introduction of new and improved treatments for the management of cancer.

We will share pre-clinical animal data with principal investigators to influence clinical trial designs. Publication of both successful and unsuccessful data in high impact journals, with the aim to publish approximately 15 scientific papers in high impact journals each year.

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

Since our approaches are applicable across a broad range of tumour types it is hoped that the drugs developed from this project licence will have wide utility across different cancer patient populations. These may not completely cure cancer patients but it expected that they will enable a significant number to live longer with improved quality of life following the diagnosis of this devastating disease.

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

New information will be disseminated at key national and international conferences via poster sessions and seminars. Publication of both successful and unsuccessful data in

relevant journals. We will share pre-clinical animal data with principal investigators to influence clinical trial designs. We work in collaboration with many scientists and groups in which we are able to share data and learning meaning that important data generated from our animal studies can be shared more widely to avoid others repeating work and to help influence other areas of animal use.

We will share good practice with AWERB and also internal in vivo community groups.

Species and numbers of animals expected to be used

- Mice: 100000
- Rats: 10500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 animal studies in mice and rats alongside many other experimental approaches and they are crucial in building up a complete picture of cancer biology. Our research using animals has helped drive advances in cancer treatment that are benefiting people with cancer all over the world today.

Our work mainly uses mice (90%), which can grow tumours which mimic those of human cancer patients. Studies of cancer in mice mimic the complex way tumours grow and spread in people with cancer. The majority of our work involves implanting a tumour on the side of the animal. Although this is unlike the patient situation the use of these models over the years has demonstrated utility in selecting viable drug candidates.

Mice can be easily genetically altered to allow us to study the genetic causes of cancer and reproduce tumour types which naturally occur in humans in the correct tissues and body systems.

We also conduct some studies in rats (10%). Some compounds that we need to test may not have sufficient levels in the blood to have an effect on the tumour in the mouse and therefore we need to use the rat as an alternative species. The rat is also usually the species of choice for toxicity studies. These studies would be conducted under a different project licence but it may be necessary to directly compare the dose level of a drug that causes an effect on the tumour to the dose level that produces unwanted side effects. This is to ensure that there is a big enough margin between activity and safety in order to progress to the clinic.

In some instances cancer growth can be influenced by hormones, these are classed as hormone- sensitive tumours. It may be necessary to run some experiments in rats that do not have high levels of circulating hormones and in these instances rats that have not yet become sexually mature will be used. These are classed as juvenile rats.

Earlier Life stages of vertebrates such as zebrafish is an option, and early studies of human xenograft models do show promise of tumour inhibition studies . However the small size and difficulty of collecting meaningful blood samples makes pharmacokinetics essential in our work, currently impossible in that model, meaning that rodent models remain the most effective model at present.

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

The rats and mice we use are bred at specialised facilities and are then transported to our facility. We primarily use breeding facilities within the UK but on very rare occasions specialised strains may be required to be imported from within the EU or USA. Animals will be transported in safe enclosed boxes that contain bedding, food, water and are maintained throughout the journey within temperature and humidity levels appropriate for the species. Upon arrival to our facility the mice or rats are transferred into clean cages. In our facility these cages are Individually Ventilated (IVC) and each cage has its own clean air, food and water supply. In addition bedding and nesting material and numerous forms of enrichment are included. Enrichment provided such as paper houses, tunnel and chew sticks allow the rats and mice to have improved welfare and demonstrate natural behaviours such as sheltering, nesting, climbing and gnawing. The cages are maintained at the appropriate temperature and humidity for the species. Cages are cleaned at least once a week and water and food is checked daily. Animals are left to get used to their new home surroundings for at least 7 days before any experimental procedures are performed.

Mice and rats are typically housed in small groups as they are social animals. On occasions animals may be singly housed e.g. on occasions male mice naturally fight with each other so may be housed singly for welfare reasons.

In a typical study animals are injected with tumour cells under the skin which grow into tumours. A wide variety of tumour types may be used within this licence. Our work is not focused on one area of cancer but across all cancer types with focus on lung, prostate, breast, ovarian, pancreatic and bladder. The majority of studies use cells that are derived from human tumours. As the human tumour tissue is foreign to the animal, the animals immune system would reject the tumour tissue and therefore we need to grow human tumours in either mice or rats that have an impaired immune system. This allows the tumour tissue to grow and not be rejected.

Tumours are usually injected as cells using a needle, this is done on the lower left or right side of the back (flank) of the animal. In some cases the tumour is not available as cells and therefore a very small piece of tumour tissue needs to be put into this area surgically. The mice or rats would be anaesthetised as this requires a very small cut in the skin so the tumour tissue can be placed under the skin. The cut is then closed using either stitches, special glue or clips. Having tumour cells injected or surgically placedunder the skin allows the grown tumour to be easily monitored and measured and does not affect the animals ability to move around.

All tumour studies will be run in a small number of animals to determine the growth characteristics of the tumour before proceeding into larger studies looking at the effect of potential anti-cancer drugs. In these small pilot studies the animals are closely observed

every day and body weights and condition of the animals are recorded to ensure that the animals are healthy. The tumour is measured at least once a week and the condition of the tumour is observed daily. If the tumour grows in a consistent manner this can then be used in subsequent studies to determine the effect of potential anti-cancer drugs.

In these studies once the tumour has started to grow the animals will start to receive doses of a potential anti-cancer drug. Anti-cancer drugs are usually dosed orally via the mouth and the animal may typically receive up to two doses per day for 28 days. The animals are closely observed every day and body weights and condition of the animals are recorded to ensure that the animals are not unwell. The effect of the potential anti-cancer treatment on the tumour growth is compared to the tumour growth in an animal which does not receive the potential anti-cancer drug. The hypothesis is that the anti-cancer drug will significantly reduce the growth of the tumour.

During the study blood samples may be taken from a vein. The blood sample is analysed to measure the level of the potential anticancer drug in the blood.

At the end of the study the animal is killed and the tumour tissue and other tissues may be taken which can then be used for further investigation.

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

The tumour is continually monitored and measured and although the tumour may continue to grow this does not appear to cause the animal any pain or discomfort and they continue to behave normally. The size the tumour can grow is limited by the use of a measurement/condition/size scoring system to ensure that it does not cause any pain or discomfort to the animal.

The dosing procedure for dosing of the anti-cancer drugs does not usually cause any issues but the drug itself may have some side effects. Side effects may include weight loss or abnormal behaviours such as being less active or not socialising or interacting with their cage mates. Strict criteria are put in place to minimise any unwanted side effects to minimise any pain, suffering or distress to the animals. The side effects usually only last for a short period of time.

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

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

Approximately 90% of animals used within the licence will be mice and 10% rats. It is expected that 90% of both the mice and rats will be returned within the moderate category and approximately 10% within the mild category.

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

- Killed

Replacement

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

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

Animals are needed in our research to help us understand the mechanisms that underpin cancer, such as the growth and spread of tumours, and to develop new ways of diagnosing, treating and preventing the disease. Cancer is a very complex disease and animal studies are essential to understand these complexities within living organisms. They are also required by regulatory authorities before any trials of new drugs can be tested in humans. Animal studies are only performed after every feasible test has been conducted on cancer cells in the laboratory and where no alternative exists.

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

Multi-cellular 'organ on a chip' models are available, but as yet have not reached the reliability and integrated multi-system complexity of the rodent model, especially when shaping the treatment of patients in the clinic. There are no immune-competent in vitro models with functionality comparable to the rodent models. Non-animal alternatives are used in the identification and selection of compounds. These generally include measurements of the drug's activity on particular target cells. Activity in particular cell types however cannot predict the activity in humans due to a complexity of issues such as availability of the drug in the body and whether it is able to reach the target cancer cell.

Why were they not suitable?

Available alternatives are not currently as good as the existing rodent models and are not as suitable because they cannot mimic the living organism and the processes that underpin cancer in a living organism.

Reduction

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

This licence reflects a well established cancer research program and the numbers of animals used within this project licence are based on the diverse areas of cancer that are being investigated.

We typically run approximately 20 studies per month, each study usually has ~100 animals per study.

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

All studies are designed to ensure that the minimal numbers of animals are used to achieve the question being asked. This is done with help and guidance from a statistician who is a maths expert who uses huge amounts of data to figure out how likely it is that something will happen or not. They ensure that all studies are designed to ensure that we are able to use the minimal numbers of animals to see an effect of a potential anti-cancer drug if there is an effect.

Good experimental design principles such as randomisation are incorporated into all experiments. All study designs are approved by a statistician.

All experiments are performed in accordance with Good Laboratory Standards (GLS). This standard sets the minimum laboratory requirements for all our research and development. This ensures that procedures and results are accurate, reliable, traceable and reproducible and where appropriate, comply with the appropriate regulatory authorities' legislation.

All experiments are performed in accordance with the PREPARE guidelines - Planning Research and Experimental Procedures on Animals: Recommendations for Excellence.

All research that will be published will be published in accordance with the ARRIVE guidelines - Animal Research: Reporting of In Vivo Experiments.

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

When different project groups want to investigate the effect of their compounds in the same tumour model wherever possible we run these within one study that share the same control group therefore reducing the requirement for multiple control groups if the studies were run independently, leading to an overall Reduction in numbers.

To minimise any side effects associated with treatment of potential drugs, a small pilot study in typically 2- 3 animals is performed to ensure the treatment does not have any unwanted side effects before progressing into larger numbers of animals.

Wherever possible multiple tumour and/or tissue samples will be taken from the same animals and may be frozen down and used in other non-animal experiments.

Refinement

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

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

We will use a wide variety of different tumour models in both mice (90%) and rats (10%). The majority of the tumours are implanted as cells in an area on the animal (the lower left or right side of the back (flank)) which allows the tumour to be easily monitored and measured and does not affect the animals ability to move around. The size the tumour can grow is limited to ensure that they do cause any pain or discomfort to the animal.

In some instances (<10%) it may be required to grow a tumour in the organ where the tumour was originally derived from, for example a breast tumour implanted into the breast tissue. The tumour may be monitored using imaging which can monitor the tumour growth.

The dosing procedure for dosing of the anti-cancer drugs does not usually cause any issues but the drug itself may have some side effects. Side effects may include weight loss or abnormal behaviours such as being less active or not socialising or interacting with their cage mates. The drugs are tested in a very small number of animals initially (typically 2 to 3 per group) and only drugs that do not have unwanted side effects can be used in larger numbers of animals.

It may be necessary to surgically neuter animals to reduce levels of hormones as the growth of some tumour models may be influenced by hormone levels (hormone-sensitive tumours). Any animals that undergo a surgical procedure will be provided with pain medication prior to the surgery (and after surgery where required) and maintained in a warm environment until full recovery to minimise weight loss. Pain medication may be administered after surgery within an edible jelly. The mice and rats will have access to a non-medicated form of the jelly prior to surgery to become accustomed to eating it.

All animals will be housed in specialised cages. These cages are Individually Ventilated Cages (IVC's) which filter the air and fully protects the animals from all micro-organisms. All food, water and bedding is also fully sterilised beforehand. All cages have various forms of enrichment included, for example a cardboard house, sizzle nest, tunnels, chew stick. The temperature and humidity is kept within a specified range that is optimal for the animals.

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

Using less sentient animals for example a non-mammalian species such as the fruit fly, is not possible since they lack a closed circulatory system and so you cannot replicate a number of the complex processes that underpin cancer such as the growth and spread of cancer.

Juvenile life stages of vertebrates such as zebrafish is an option, and early studies of human xenografts into zebrafish models do show promise of tumour inhibition studies (Xaio et al 2020). These models rely on protected life stages of zebrafish, and may need larger numbers, although moving to a vertebrate considered less sentient than a rodent may be a significant Refinement in future (Costa et al., 2020). However the small size and difficulty of collecting meaningful blood samples makes the pharmacokinetics essential in our work, currently impossible in that model, meaning that rodent models remain the most effective model at present. Others have investigated embryo larval life stages of zebrafish that would be unprotected within the EU (since they work before the day 5 threshold of

protection) with some success, but reliable quantification and exposure through internal concentration remains the barrier in this case as well (Hill et al., 2018).

Costa et al 2020 EBioMedicine (The Lancet) https://www.sciencedirect.com/science/article/pii/S2352396419307881 Developments in zebrafish avatars as radiotherapy sensitivity reporters — towards personalized medicine.

Hill et al. 2018 F1000Res https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6234738/

Xaio et al. 2020 Trends in Cancer (Online 17th April) https://www.sciencedirect.com/science/article/abs/pii/S2405803320301217

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

All animals will be acclimatised for 7 days from arrival before they undergo any experimental procedure.

We closely follow and implement the latest welfare guidelines and therefore handle animals in a way that causes the least amount of harm or stress to them as possible while conducting these experiments.

All surgery is performed in concordance with 2017 LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

Explore if there are options to apply any palliative treatments to minimise any adverse effects of dosing. Explore use of refined dosing applications in collaboration with the NVS, for example use of pin-port.

We use a new needle for every individual animal injected parenterally. The same applies for when we implant cells.

Use of banked animal tumour and/or normal tissue samples taken from previous studies which can be used in non-animal experiments.

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, P., Aboagye, E., Balkwill, F. et al. Br J Cancer 102, 1555–1577 (2010))

Animal research: Reporting in vivo experiments: The ARRIVE guidelines. Br J Pharmacol. 2010 Aug; 160(7): 1577–1579.

Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18(7): e3000410. https://doi.org/10.1371/journal.pbio.3000410

PREPARE: guidelines for planning animal research and testing (Adrian J Smith, R Eddie Clutton, Elliot Lilley et al. Laboratory Animals Volume: 52 issue: 2, page(s): 135-141

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

I actively participate in the in vivo community, attending and presenting at conferences relevant for oncology preclinical models. I also follow the NC3R's website to keep myself updated on relevant 3R's initiatives (https://www.nc3rs.org.uk/news/using-award-scheme-promote-3rs-innovation). We also actively discuss and implement new 3R's initiatives and run a yearly 3R's competition, sharing information globally across different establishments. We actively set annual Refinement goals, for example alternative mouse handling.

23. Central circuits underlying visceral and somatic sensations

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Somatic pain, Visceral pain, Chronic pain, Neuropathic pain, Spinal cord

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?

The aim of this project is to identify distinct nerve cell circuits in the spinal cord that are involved in processing sensory information from the skin, joints, and internal body organs under normal conditions, and to determine how the properties of these circuits change in long-term pain states.

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

Why is it important to undertake this work?

Our bodies have evolved complex circuits of interconnected nerve cells (neurons) to relay information from our skin, muscles, joints, and internal body organs up to the brain for perception of various sensations including touch, temperature, and pain. The information that gives rise to these sensations is relayed to the brain along distinct sense-specific pathways that pass through the spinal cord. In the spinal cord, dedicated collections of neurons form functionally specific circuits that play a crucial role in helping our central

nervous system (CNS) make sense of the barrage of sensory information it receives. For each type of sensation, dedicated neuronal circuits act to prioritise which signals are sent to the brain for perception. These circuits also play important roles in refining the sensory information being processed by our CNS, helping ensure that we mount responses that are contextually relevant. Understanding the cellular make-up of these complex and highly variable neuronal circuits presents a major scientific challenge in our efforts to understand how we respond to our environment under normal conditions. This challenge is compounded when we try to understand how these systems change in diseased conditions that lead to altered sensory perception and chronic pain states.

Chronic pain affects approximately 20% of the global population and is often resistant to any form of treatment. This debilitating condition can lead to patients experiencing heightened levels of pain perception, spontaneous pain, or the perception of pain from stimuli that were previously innocuous (e.g. light touch or brushing of the skin now being perceived as painful). Since changes in the activity of certain nerve cells in the spinal cord is known to play a key role in the development of these chronic pain states, it is vital that we identify precisely which circuits (and which cellular components in particular) change in diseases conditions so we can then develop targeted approaches for the development of more effective pain management strategies. To do this, we must first study how these distinct circuits function under normal conditions and then determine whether (and if so, how) each neuronal component within these circuits change in the various chronic pain states. We have previously identified two distinct sub-populations of dorsal horn neurons and shown that each contribute to different aspects of chronic pain: the loss of normal function of a population of inhibitory interneurons leads to heightened touch-evoked pain, whereas abnormal recruitment of a population of excitatory interneurons leads to increased pain perception. To help develop more effective treatments for a variety of chronic pain states, we now aim to identify other neuronal populations that also contribute to these pathological conditions, determine how/whether their contributions to the circuits they are part of change, and devise ways of restoring their normal function.

In this project, we aim to determine how distinct populations of nerve cells contribute to processing sensory information under normal conditions and establish whether these change in chronic pain states. To achieve this, we will use a series of transgenic mouse lines that allow us to target and manipulate the function of several distinct neuronal populations with precision. Some of these lines will generate mice in which intrinsic fluorescent proteins will be expressed exclusively in our targeted population(s), helping us identify these cells for electrophysiological recording experiments and histological analysis. Other lines will generate animals where specific light- or drug-sensitive channels are expressed selectively in our cells of interest, providing us a means of manipulating the function of these neurons with precision, and in doing so helping us to establish their respective roles in different behaviours. We will then use experimental models of different chronic pain states induced by either surgical procedures or following direct manipulation of our target neuronal populations to determine whether (and if so, how) the functional properties of these cells change. By targeting and manipulating cell populations selectively and with precision, we can determine their respective contributions to both normal and pathological pain processes. It is only possible to conduct these experiments in animal

models, but the findings of these studies will identify novel targets for alleviating chronic pain states in pateints.

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

Chronic pain is a debilitating condition that is often resistant to treatment. The need to develop more effective treatments to address this unmet clinical problem is evident, but one of the major challenges we face in addressing this problem is our limited understanding of the neuronal processes involved in perceiving pain sensations.

The principal aim of this project is to identify distinct populations of nerve cells in the spinal cord that are involved in how we perceive pain. By identifying these cells, and understanding how they contribute to sensory perception in normal and chronic states, the results of these studies will be important in helping develop new, more specific targets for interventions to alleviate a variety of pain conditions.

The results of these studies will be published in international peer-reviewed journals and presented as talks or conference presentations at academic gatherings and to pharmaceutical companies. Our findings will help other pain researchers and pharmaceutical companies identify novel targets for the development of new pain management therapies and analgesics.

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

The findings of these studies will be of direct benefit to other scientists working on the somatosensory and visceral systems, as well as those in the pharmaceutical industry, particularly in relation to development of new analgesic drugs.

The ultimate beneficiaries of this collective effort to devise more effective pain management approaches will include human patients and animals suffering from chronic pain, and the clinicians responsible for their treatment. Chronic pain affects approximately 20% of the global population, however, one-in-three patients remain resistant to any form of treatment currently available. The personal impact of chronic pain is widespread and significant: 65% of these patients reporting difficulty in sleeping, 49% develop depression and 16% claim "the pain is so bad that they want to die".

Approximately one-in-five chronic pain patients consider suicide, and between 5 and 14% of these patients attempt to take their own life. The need for more effective pain management therapies is clear, and the work outlined in this project will contribute significantly to addressing this clinical need.

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

The findings of these studies will be published in peer-reviewed journals and presented as talks or conference presentations around the world at academic gatherings and to pharmaceutical companies. The dissemination of this information will allow me to bolster existing international collaborations, as well as help develop new partnerships with both academics and pharmaceutical companies. This information will also be disseminated to the general public through both patient and public involvement and engagement (PPIE)

Events, as well as outreach projects. The development of more effective pain management strategies will have a significant impact on improving the health and well-being of chronic pain patients, and consequently that of their families. Improved treatments for chronic pain will also be of considerable economic benefit to society due to the Reduction in time lost from work.

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 aim of this project is to determine the roles of different nerve cell populations in processing sensory information from the skin, joints or internal organs. Because of the need to match detailed information about both the anatomy and the physiology of targeted neuronal populations with assessment of behavioural responses to manipulation of these same cells, work of this type can only be performed in experimental animals. The mouse is the only species that could be used in this project because of the availability of genetically altered lines in which fluorescent proteins or recombinases are present in specific neuronal populations. These transgenic mice allow us to manipulate the activity discrete components of highly complex sensory pathways using experimental approaches. By manipulating these neuronal targets specifically under experimental conditions, we can determine the role they play in the development of chronic pain states. In some cases, we will administer transgene inducing agents (e.g. tamoxifen) to neonates or pregnant females. This is necessary as it provides the only means of capturing the expression of markers that may only be expressed at early stages of embryonic development. Most experiments will be conducted in juvenile/young adult mice given that the neuronal circuits in the central nervous system are fully established by these stages. This provides us with a means of comparing functionally distinct circuits in naïve and chronic pain states which will help identify how these change in pathological conditions. These changes are likely to underlie the development of the resultant pain states and will provide novel targets for the development of new pain management therapies.

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

Transgenic mice will be bred under this project for use in studies to further our understanding of how sensory information derived from the skin, joints, and internal organs is perceived under normal and pathological conditions. In this project we will target discrete neuronal populations using molecular-genetic and surgical approaches to establish their role in processing sensory information.

This project employs four protocols that will allow us to address our scientific questions:

Protocol 1; Breeding and Maintenance: This allows us to generate appropriate transgenic mouse lines for use in all subsequent experiments. Animals used in this protocol will typically be young, sexually mature mice less than one year in age.

Protocol 2; Neuronal Tracing: This provides a means of labelling specific neuronal populations using tracer injections (under anaesthesia) or molecular-genetic approaches for use in subsequent anatomical or electrophysiological studies. Animals used in this protocol will typically be young adult mice (of both sexes) and less than four months in age. Animals used under this protocol may be subjected to a maximum of three steps before experiments are ended, or two steps before being transferred to either Protocol 3 or 4.

Protocol 3; Somatic pain models: All animals used under this protocol will undergo recovery surgical procedures under general anaesthetic to either set up models of neuropathic or post-operative pain.

Some animals will undergo implantation of light-emitting diodes that will allow us to control the activity of distinct populations of nerve cells. These animals will then be used in behavioural testing studies (either awake or under terminal anaesthesisa) to establish their responsiveness to various forms of cutaneous stimulation. Animals used under this protocol will typically be young adult mice (of both sexes), and will undergo no more than three of the steps outlined. All experiments will end no later than four weeks after surgery to induce neuropathic or post-operative pain states.

Protocol 4; Visceral pain models: All animals used under this protocol will undergo manipulation of internal body organs to mimic visceral pain states. Some animals will undergo implantation of light- emitting diodes that will be used to control the activity of distinct populations of nerve cells. These animals will then be used in behavioural testing studies (either awake or under terminal anaesthesisa) to establish their responsiveness to various forms of visceral stimulation. Animals used under this protocol will typically be young adult mice (of both sexes), and will undergo no more than three of the steps outlined. All experiments will end within three weeks of surgery to implant light-emitting diodes.

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

Given that we are studying the neuronal basis of chronic pain states, it is unavoidable that animals used in this project will experience moderate pain. In many cases, this pain is transient, but may last up to a maximum of 18 weeks. In most cases, experiments will be conducted on animals under general anaesthesia to minimize the impact of these pain states. We are aware of the potential for adverse effects associated with the use of these various models (e.g. weight loss, self-directed tissue damage and locomotor impairment), but incidence of such events will typically be very low (<3%). These experimental approaches are established locally, and research and technical staff are well-versed in spotting these potential problems through a careful post-operative monitoring program.

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

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

Mice used in Protocols 2, 3 and 4 (excluding those on Protocol 1) will experience mild (~30%) to moderate (~70%) levels of pain. Where possible, the pain states in these experimental animals will be managed through the administration of analgesics and close monitoring to minimize undue suffering.

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

- Killed
- Used in other projects

Replacement

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

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

To achieve our scientific aims, we must study functional neuronal circuits in the spinal cord. This is achieved by matching detailed anatomical analyses of neuronal populations, with behavioural readouts of activity within defined neuronal circuits and physiological data generated from tissue preparations derived from transgenic mice. These experimental approaches, which are wholly dependent on being able to target and/or manipulate discrete neuronal populations, are only achievable through the considered use of animal models.

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

This project assesses the functional impact of distinct neuronal populations on behaviour and must therefore be conducted on live animals. It is not possible to determine the functional significance of neuronal circuits without being able to correlate the anatomical and electrophysiological properties of individual neuronal components with the induction of behavioural responses following targeted manipulation.

Why were they not suitable?

- Not applicable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 target discrete neuronal populations repeatedly and with precision, we will need to breed several distinct transgenic mouse lines for our experiments. These breeding programs will produce animals we can use to visualise and manipulate the activity of distinct circuits specifically. The precision of these approaches ensures our experiments are reproducible, and this leads to a substantial Reduction in the numbers of animals required to generate statistically meaningful datasets than would otherwise be possible.

The intersectional genetic strategies we use to target these neuronal populations require the crossing of several lines, e.g. to generate triple- or quadruple transgenic lines where different recombinases as well as different reporter lines are expressed. Some of the mice we will have to use must be heterozygous, and in complicated crosses, this results in a low yield of viable offspring.

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

Where possible, we have developed breeding strategies that will generate homozygous mice for use in subsequent crossed to produce offspring with the appropriate genotype. It is possible that some offspring of particular genotypes can be re-used in breeding programs to maintain lines.

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

We employ strict quality control measures over all experimental aspects to optimise the number of animals used in these projects, such as genotyping and phenotyping all litters to validate animals designated for continued use. Where possible, tissue is collected at the end of procedures so it can be used in parallel anatomical studies.

Refinement

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

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

These studies can only be performed on mice, due to the wide range of genetically modified lines available for this species. Animals that undergo surgical procedures under this protocol will show a moderate level of post-operative discomfort for a short period. This is inevitable, but the molecular-

genetic targeting approach we have adopted ensures we use fewer animals than would otherwise have been necessary if we were to use more traditional approaches. These traditional approaches generally involve very crude manipulation of large groups of neuronal populations and lack precision of selectivity to defined subpopulations in distinct circuits (e.g., systemic administration of drugs to study neuronal circuits in the spinal dorsal horn will also affect circuits in other parts of the central nervous system).

The novel approaches we will use in this project allow us to target and manipulate discrete neuronal populations with greater precision than previously possible. This allows us to influence activity of neurons in defined circuits specifically from which we can determine the functional significance of our targeted population. We can control these populations in awake animals with great precision in a number of ways, with the resultant effects lasting for variable periods: optogenetic approaches using light pulses directed to appropriate body regions changes neuronal activity instantaneously and the resultant effects on behaviour last a matter of seconds; chemo genetic approaches, where administering highly specific designer drugs to activate/inactivate neuronal populations, allows us to influence neuronal activity for periods of up to two hours; genetic silencing approaches allow us to block communication relayed through distinct neuronal populations permanently, without killing the cells; genetic ablation approaches allow us to eliminate distinct neuronal populations selectively. These approaches provide us with the means of dictating the activity of distinct neuronal populations (and consequently the circuits they contribute to). By limiting/controlling the activity of distinct neuronal populations using these approaches, we can establish their functional significance under normal and diseased conditions whilst also minimizing any pain, suffering, distress, or lasting harm to animals under our care. These approaches also provide a consistent means of targeting distinct neuronal populations (and circuits) with great precision, ensuring greater experimental reproducibility and an increased return of data.

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

Given that we intend to study changes to mature spinal circuits under pathological conditions, most of our studies will be in juvenile and young adult mice. It is not possible to carry out these studies in animals that are still developing since compensatory changes at these early stages may mask structural reorganisation seen in the adult nervous system. Nonetheless, we will use animals at embryonic and neonatal stages to label cells early in development but will only look to target and manipulate these cells in mature animals.

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

All experimental procedures are reviewed periodically to ensure optimal performance, in consultation with other local groups, NVS and the area Home Office Inspector to ensure best-practice and optimal performance. All of the experimental procedures outlined in this project are established in our group, and are the most refined approaches to address our scientific questions whilst also ensuring minimal pain and discomfort. For example, by defining unique molecular profiles for the neuronal populations of interest, we have developed intersectional genetic strategies to target these cells specifically. Furthermore,

by adopting chemogenetic and optogenetic approaches to manipulate neuronal populations only transiently, our optimised procedures greatly reduce the experimental impact on our animals.

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

We will perform all studies in line with guidance found on websites managed by NC3Rs and the local institution, as well as in the published literature.

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

I will follow directives from our area Home Office Inspector, local NVS, and updates from NC3Rs directly (www.nc3rs.org.uk/the-3rs), as well as keeping up-to-date with best practices as described in the scientific literature.

24. Immune protection to influenza virus

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

influenza virus, lung, vaccine, T cell, immune memory

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Following an influenza virus infection, your body builds up specific defences to protect you from future infections. We aim to understand how different cells in the lung, including immune and structural cells, communicate to provide this protection to multiple different viral strains following an influenza virus infection or vaccination.

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

Why is it important to undertake this work?

Influenza virus is a prominent human pathogen. It has caused multiple world-wide outbreaks over the past several 100 years and is an annual seasonal disease. Influenza infects the airways and lungs. In certain individuals, the infection causes severe pneumonia, which can lead to death. Annually, there are around 4 million severe cases and approximately 400,000 deaths from influenza virus. In the UK, 1000-3000 people are admitted to intensive care units annually and around 5% of these individuals die as a consequence of the infection. It is estimated that in high income countries, seasonal

influenza infection cost €56.7 million per million people. The cost of a global pandemic that could, like COVID19, paralyse countries, would be considerably more.

Influenza virus infection educates the body's immune system leading to the formation of immunological memory. This means that the immune cells that respond to influenza virus can respond more quickly and more effectively to a second infection with the same virus. This immunological memory is the basis for the success of vaccines that also educate immune cells that protect the body from infection.

Current influenza virus vaccines are much less effective at generating long term immune protection compared to an actual infection with the virus. We think that this is because the vaccines are less efficient than the virus at educating a particular type of immune cell called T cells. T cells that are educated to respond to influenza virus are important as they can protect against lots of different types of influenza virus. This contrasts with current vaccine derived immune protection that leads to the generation of antibodies that, because of mutation of the viral surface proteins, are only able to protect against that season's influenza virus variant. T cells can provide broader protection to many different strains as they recognise parts of the virus that are conserved across many different strains.

We have only a limited understanding of the cells and molecules that are required to direct the development of long lived educated T cells following influenza virus infection. We also have an incomplete understanding of how the educated T cells protect against subsequent infections, and what other cells in the lung work

What’s the aim of this project?

Following an influenza virus infection, your body builds up specific defences to protect you from future infections. We aim to understand how different cells in the lung, including immune and structural cells, communicate to provide this protection to multiple different viral strains following an influenza virus infection or vaccination.

Why is it important to undertake this work?

Our work will address which cell types in the lung are involved in generating longterm protective immunity following influenza virus infection. This is important as it could highlight which cells vaccines need to act on to ensure longterm protection to different influenza virus strains. We will also examine whether newly identified parts of influenza virus that are highly conserved across most influenza virus strains, stimulate an immune response and can provide immune protection. These studies may help identify novel parts of influenza virus that can be used in a 'universal' influenza virus vaccine.

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

Our studies will generate new information related to the immune response to influenza virus infection. This information will be shared with the scientific community through oral and poster presentations at national and international meetings and through publications that will be free for all to view (open- access).

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

In the short-term, the main beneficiaries of our work will be other scientists as our data adds to a body of knowledge about the immune response to influenza virus. We will communicate our new knowledge by presentations at scientific meetings and via publications in scientific journals. These data will help to move the field forward and generate new hypotheses relevant to how our bodies fight infections.

In the longer term, this collective body of work will help scientists to design novel vaccines that can generate local immunity within the respiratory tract and lung to many different strains of influenza virus. Improved vaccines for influenza virus would reduce the number of serious diseases and deaths caused by this virus. The research may also be more widely applicable to other respiratory viruses, for example, respiratory syncytial virus and SARS-CoV-2.

These longer term impacts of our research have social and economical benefits too. Vaccines are one of the most cost-effective forms of health care. New, more effective vaccines will reduce the number of people who become ill following infection, reducing the number of people who take time off work and/or require hospital treatment, or who die as a consequence of the infection.

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

We will maximise the outputs of our research by ensuring that as much of our data as possible is published, either in primary scientific articles or via publications that are methods focussed. We will also share our data at national and international scientific conferences through oral and poster presentations. We will also share our methods and data in discussions with former and current colleagues, collaborators, and will provide detailed step-step methods to any researchers who request this.

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.

We use mice for our experiments for a number of reasons:

They are phylogenetically the least complex mammal with similar immune system to humans. This means that we can extrapolate our data to understand what might happen in human disease.

They have been used in many similar studies to ours. This means that we can make sense of data in the context of the wider field and accelerate our understanding of disease.

There are many genetically modified mice available. This means that we can ask very precise questions that enable us to understand which cells and molecules are involved in the disease process.

In our studies we use adult mice to examine how a mature immune system responds to viral infection.

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

Typically, mice in our studies will be infected with influenza virus. We will then examine the immune response in the lungs and in immune organs in the subsequent days to weeks. In some studies, we will give the mice a vaccine and examine whether they are protected from a subsequent infection with influenza virus. In other experiments, we will manipulate cells and molecules in the lung either before or after influenza virus infection to determine what role these cells and molecules play in protection the host.

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

Mice infected with influenza virus are likely to lose weight; rarely they will show other clinical features (hunched, reluctance to move, isolation and failure to groom). Typically weight loss begins 6-7 days following infection and mice have regained their starting weight by day 12-14 after 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)?

- Subthreshold: 10%
- Mild: 20%
- 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?

Although studies using cells grown in the lab can recapitulate some aspects of the interactions between the different cells we study, they cannot adequately model the complete and complex array of immune responses involved in the generation of protective memory response against a pathogen. The many interactions between the cells of the

immune system, lung structural cells and with the infectious agents cannot be fully modelled by cells grown in the lab. Therefore, further animal studies are required.

Mice are used in this project as they represent the least complex vertebrate group where the influenza virus infection model has been developed. Moreover, the transgenic and knockout models, which will allow us to understand how the cells we study work, are only available in mice.

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

For immunological studies, the only non-animal alternatives open to us are using cell lines that have already been established, using human cells, or using data have already been generated in mathematical based models.

Why were they not suitable?

The aim of our studies is to examine how cells within the lung change following influenza virus infection in the short (days) to the longer term (months). Some of the cells we examine can move into and from the lung over this time course and therefore our studies require an intact circulation system. Our studies examine the long term effects of communication between different cells in the lung. This includes immune cells, such as T cells, and structural cells, including epithelial cells and fibroblasts. The communication of these populations is dependent on their precise location within an intact, living organ over the days, weeks and months following infection.

For these reasons, current in vitro techniques using isolated cell types or artificial organs grown in vitro are not able to replicate the complex dynamic relationships of the different cell populations that we study in the intact animal.

Current cell lines do not accurately represent the immune and structural cells that we examine. In the main this is because the cells we study are heterogenous populations while cell lines are, by nature, non-heterogenous, and because cell lines have been treated so that they can survive in culture. This artificial cell life-time means these cells do not act in the same way as cells from an animal. Human cells are not an option for us as we examine lung cells at defined times after influenza virus infection. Finally, there is not currently enough information about the consequences of longterm communication between immune and structural cells for us to construct accurate mathematical 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 practicesthat are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

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

Within an experimental system we need enough animals to be certain that any changes we observe between an experimental group and a control group reflect real differences. If we want to examine whether a particular cell type has changed following influenza virus infection and whether these changes help protect the animal following a subsequent infection, we will need to set up two different types of experiment.

To address the first question, we will need to examine the cell type of interest in animals that have and have not been infected. Here we need around 4-5 mice in the two groups to give us confidence in any difference that we observe. This number is based on the level of variance that we see in uninfected and infected animals in previous similar types of experiments over the last 19 years and similar to numbers used by other researchers who use influenza virus infection in mice. We also do these experiments at least one more time to make sure our results are consistent and reliable. This is standard practice and we would not be able to publish our results in scientific journals without doing this.

Experiments to test whether any changes to a particular cell are involved in protecting the animal from a subsequent infection are more complex. Here we typically need around 10-12 animals per group as we see more variation in these studies. We also need more groups to ensure that our experiment has worked as we expected. For example, we need to treat a group of animals in a way that we know will protect them from infection, and/or to have a group in which the cell type or interest is present and one in which the cell type is removed. These groups are required to give us confidence in our data.

The number of mice needed are also based on the different time points we will examine following influenza virus infection. We are interested in how immune and structural cells change in the lung change following influenza virus infection and for how long these changes persist.

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

When planning experiments, we take into account guidance from NC3Rs through the experimental design assistant. For experiments that involve new reagents or new analysis, we seek guidance from colleagues with experience in these areas.

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, we will perform pilot experiments. This includes when we are using new chemicals or have a new way to analyse our data. These pilot experiments will contain fewer mice and fewer groups as their aim is to test the chemical or the experimental read out rather than address a scientific question. This will ensure that when we perform the full experiment, we are confident that our reagents or experimental read outs will provide us with reliable data.

In some cases, when the conditions and timing of the pilot experiment are exactly the same as the full experiment, we will be able to include the data from the pilot experiment in

the full experiment. This will enable us to reduce the number of animals used in some of the control groups in the main experiment.

As many of our objectives require tissue from mice at different time points following influenza virus infection, the researchers will be able to share tissues to address their own specific 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.

In many of our experiments, we will infect mice with influenza virus. This is essential for our project which is aimed at understanding how cells in the lung change following an infection and how these changes lead to protection from a subsequent infection. The infection will lead to weight loss lasting from about day 6 to day 10 following the infection. Additionally, weight loss is a readout of immune protection in our infection challenge studies.

We ensure that the dose of virus that we use for these studies is sufficient to drive a consistent immune response but is low enough so that it does not cause more suffering or distress than is necessary. If we used a lower dose of virus, we would risk some animals clearing the virus without the induction a strong and persistent immune response - i.e. in these animals no adaptive immune response against the virus would be generated and we would not be able to use these animals in our studies. This would mean more mice would need to be infected to ensure we had sufficient animals who did have a strong and persistent immune response to use in our studies.

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

The major aim of our project is to understand how different cells in the lung communicate with each other during and following an infection. We need, therefore, to examine the lung immune and structural cells at different times following infection. We use adult mice, as younger mice do not have fully developed lungs. Mice are most appropriate animal to use for our studies as their immune systems are similar to humans and we have a great deal of knowledge about immune responses in mice, helping us to put our data in the context of a wider field and accelerate new understanding.

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

Mice on our procedures are monitored when we expect them to show signs of ill health. This occurs following infection with influenza virus and we monitor them by weighing and by observing their behaviour and appearance. Typically, we carry out this monitoring between days 4 and 12 following infection. This is based on our extensive use of the influenza virus infection model. Should we find that infected mice begin to lose weight before day 6, we will begin monitoring then from day 2 rather than day 4 of infection. Mice that fail to regain their starting weight will be weighed until they do, usually by day 20 post-infection.

We euthanise mice if they lose 20% of their starting weight or show unexpected clinical signs, including reduced movement. Animals that fail to regain their starting weight 40 days following infection are also euthanised as a failure to re-gain weight would suggest ongoing clinical disease.

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

We follow guidance from 3R and the ARRIVE guidelines and any additional local guidelines within our establishment.

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

Up to date guidance will be monitored via the NC3Rs website (http://www.nc3rs.org.uk/) and through communications from our animal facility.

We keep up to date on relevant scientific literature using the influenza virus infection model in mice. Should there be relevant and feasible modifications to the current methods that would enable us to reduce the numbers of animals we use, we would first test whether these altered our main read outs of disease and immune response, and if our main findings could be replicated, adapt these modifications.

25. Neuronal Control 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, Energy metabolism, Potassium channel, Oxidative stress

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

Sleep is one of the great biological mysteries. Each night we disconnect ourselves from the world for seven or eight hours—a state that leaves us vulnerable and unproductive. But despite these risks and costs, we still don't know what sleep is good for. Whatever sleep's purpose is, however, is clearly essential: chronic sleep loss shortens life.

Studies in fruit flies have revealed that the need to sleep is tied to the efficiency with which brain cells (and perhaps other parts of the body) burn the food we eat. The proposed project will test whether the same sleep-regulatory mechanism also operates in mice. The work is expected to advance our understanding of the regulation and function of sleep and identify new strategies for the treatment of sleep 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?

Sleep disturbances are among the most common medical problems. A large majority (75%) of adults in Western societies report at least intermittent sleep disruptions; up to a quarter suffer from persistent daytime sleepiness; 10% experience chronic insomnia.

Sleep disturbances contribute to a number of medical, psychiatric, and neurological conditions and are a leading cause of traffic accidents. Diseases associated with impaired sleep include diabetes and obesity, mood disorders, and many neurodegenerative diseases, including Alzheimer's and Parkinson's.

Although sleep medications are among the most commonly prescribed drugs, the value of the prescription drug market for sleep indications has declined sharply in recent years as patents have expired. This decline is a vivid testament to the failure of the pharmaceutical industry to materialise innovation, despite significant risks and adverse effects of existing drug treatments.

The importance of the proposed project is two-fold: the work will aim to define a novel mechanism connecting energy metabolism and sleep, and it will seek to harness this insight to devise rational new strategies for therapeutic intervention.

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

New information. The proposed work will investigate the role of potassium channel β-subunits—and, in particular, their oxidoreductase activity—in the regulation of mammalian sleep. It will determine the identity and sleep-relevant sites of action of potassium channel β-subunits in the brain and determine whether the administration of β-subunit substrates induces sleep in mammals. Together, these studies promise to lay the foundation for a rational new approach to the treatment of sleep disorders, with potentially significant health and economic benefits.

Scientific publications and public engagement. We will disseminate our findings to biomedical researchers, health professionals, and the general public.

Training of graduates and postgraduates. Its hypothesis-driven agenda, rigorous mechanistic outlook, wide methodological range, and considerable application potential make the proposed programme an excellent training opportunity for junior colleagues.

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

Researchers in many different disciplines will benefit from the results of the proposed project.

Neuroscientists, biochemists, and cell biologists, because our work connects different aspects of their fields and in a surprising, seamless fashion: the regulation and function of sleep, mitochondrial electron transport, lipid and redox metabolism, the biophysics of action potential generation, and the regulation of enzymatic catalysis and ionic conductivity.

On channel biophysicists, because the identity of the endogenous molecules that serve as substrates for potassium channel β-subunits and couple a cell's excitability to its metabolism remains an important open question.

Biomedical researchers, because energy expenditure ('rate of living'), oxidative stress, and sleep have been implicated repeatedly and independently in ageing, lifespan, and degenerative disease. Our work aims to generalize and solidify an underlying mechanistic connection suggested by studies in fruit flies.

Pharmaceutical researchers and clinicians, because our work formulates and tests a new concept for the management of sleep disorders.

In the long run, if this concept for the management of sleep disorders is validated and introduced into clinical practice, there may also be medical and economic benefits.

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

In addition to sharing our findings through standard academic channels (preprint servers, peer- reviewed publications, conferences, and seminars) and social media, we will ensure that significant discoveries are accompanied by press releases and short video clips intended for non-specialist audiences.

We will also rely on media contacts to communicate the health implications of our research and any attendant lifestyle advice (provided, of course, that the advice is based on solid scientific evidence and there are no conflicting intellectual property considerations).

Species and numbers of animals expected to be used

- Mice: 19500

Predicted harms

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

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

Previous research in the fruit fly has shown that energy metabolism, oxidative stress, and sleep—three processes implicated independently in lifespan, ageing, and degenerative disease—are mechanistically connected. The crucial link consists of redox-sensitive potassium channel β-subunits that regulate the electrical activity of sleep-promoting neurons. Given the great fundamental and clinical importance of sleep, and the potential of KVβ substrates to define novel prototypes of sleep-regulatory drugs, it is important to test whether the same sleep-control mechanism also operates in mammals.

Because the use of mammals is integral to the question at hand, we will use mice, the phylogenetically lowest, commonly used mammalian laboratory species with a brain sufficiently large to accommodate the recording devices required by this project. Techniques for chronic neuronal recordings, the analysis of sleep stages, and viral and germline genetic modification are well established in this species.

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

Typical procedures involve injection of viral vectors into specific regions of the brain, implantation of recording devices, and long-term recording of brain activity for days to weeks. A minority of mice will enter longevity analyses.

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

Expected adverse effects include the consequences of genetic modifications (sleep impairment, increased probability of seizures, and reduced lifespan), stress from single housing, side effects of pharmacological interventions (weight loss), and rare complications from surgery. The adverse consequences of genetic modifications are likely to last from early adulthood to death unless successfully counteracted by compounds discovered during this project. Stress due to single housing and other protocol-related effects (e.g., weight loss) will last for the duration of the experimental intervention.

Expected severity categories and the 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 = 9000 Moderate severity = 10500

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 behavioural state that can only be defined and investigated in intact animals. While invertebrates, especially the fruit fly Drosophila, are a powerful model for fundamental research, the explicit aim of the present project is to probe the extent of functional conservation of sleep- regulatory mechanisms between insects and mammals. The use of a mammalian species is therefore a sine qua non.

Which non-animal alternatives did you consider for use in this project? Computer models, cell cultures, and phylogenetically lower model organisms.

Why were they not suitable?
Sleep cannot yet be modelled in computers or cell cultures; testing the hypothesis in phylogenetically lower model organisms than the fly, such as the nematode, would defeat the very purpose of this project.

Reduction

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

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

Our estimate is based on a Mendelian ratio of inheritance for the generation of homozygous mutant mice that includes a 10% contingency. Because a minimum of 6 mutant lines must be tested, breeding and maintenance accounts for the majority (13,000) of the total estimated number of animals.

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

Our experimental designs take advantage of randomization, allocation concealment, automated data analysis, and blinding to prevent subjective bias, and of within-animal designs to minimize the total number of animals to be used. Only genetically modified lines showing relevant abnormalities (e.g., sleep impairments and/or sleep- related symptoms) will be continuously maintained, while those with non-relevant abnormalities will be discontinued as soon as possible.

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

Complex surgeries that require precise targeting will be first extensively practiced on cadavers to reduce both variability and the total number of animals used. Breeding will be done in an efficient manner and closely monitored so as to prevent overproduction. Genetically modified mouse lines that do not, upon rigorous testing, show robust sleep phenotypes will be cryopreserved and discontinued. Where applicable, within-subject experimental designs will be employed so the animals can act as their own controls.

Refinement

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

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

Mice will be used for this project. This model is the most appropriate to address the Objectives of this project.

Various state-of-the-art methods, such as stereotaxic cranial surgery and electroencephalography will be used to target specific regions of the brain and record brain activity as an animal sleeps. Given that no alternative, more refined methods exist to address the Objectives of this project, the methods selected will cause the least pain, suffering, distress, or lasting harm to the animals during the course of this research.

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

Animals at a more immature life stage cannot be employed because only adults can wear the recording implants required for this research. All background research for this project derives from work in "less sentient" species, but the purpose now is to test the generality of what was found in the fruit fly in mammals. The mouse model is the least sentient animal that can be used to address the Objectives of this project.

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

Animals will be monitored daily and intensively following any advanced procedures, and extensive post-operative care such as pain management will be rigorously applied to minimize welfare costs. The earliest humane endpoints consistent with the scientific goals will be applied.

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

The NC3R's guidance to best practice will be followed to ensure the 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?

Regular attendance of meetings that address needs and updates of advances in the 3Rs; subscription to newsletters and bulletins; discussions with colleagues and staff.

26. Investigating Sex-Based Differences in 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Sex Chromosomes, Sex Hormones, Autoimmunity, Inflammation, Immunology

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to investigate how sex hormones and sex chromosomes affect immune system function during health and inflammatory disease.

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

Why is it important to undertake this work?

Despite the appreciation that immune responses differ in males and females, using sex as a biological variable in experimental studies does not routinely take place. In addition, not all published animal studies report the sex of the animal used during in vivo experiments and experiments with isolated cells rarely report the sex of the donor. This means that in many situations it is not known whether the reported effect is applicable to both male and female immune systems. Greater understanding of the mechanisms by which the immune

response varies between males and females may allow the development of better targetted therapeutics, especially in disease where there is a noticeable sex-bias such as lupus and arthritis.

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

The outputs for this project will include greater insight into the how the immune system differs in males and females, leading to publications and in the future better ways to treat male and female patients more targetted manner.

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

The short-term benefits of this project (within the next five years) will include greater insight into the how the immune system differs in males and females. This information, which we intend to publish in peer- reviewed journals and report at conferences, will inform the work of researchers who work on related projects where there is also a sex bias in disease onset/outcomes (such as Lupus, COVID-19, certain cancers and sepsis). With the emergence of the concept of personalised medicine, it is both important and timely to consider the mechanism underlying the differences in observed pathophysiology of disease between males and females.

In the long-term (>10 years), our research has the potential to benefit the management of disease in patients with lupus and rheumatoid arthritis by obtaining a greater understanding of the cause of these complex diseases and providing new therapeutic strategies. Rheumatoid arthritis is a highly debilitating disease that affects 0.8% of the UK population and results in a reduced life expectancy. In 2010 it was calculated that rheumatoid arthritis alone cost the NHS £560 million/year. The cost to the wider economy of rheumatoid arthritis was calculated at £1.8 billion in terms of sick leave and work-related disability. Lupus has a lower prevalence in the UK population than arthritis, affecting around 28 in every 100,000 people in the UK, however it is also a potentially fatal disease; of the 396 patients we have followed over the past 25 years, 14% have died, with an average age of 57 years. While the economic burden of lupus to the economy as a whole has yet to be assessed, in 2000 it was estimated that direct treatment costs/patient/year to the NHS were £7913, equivalent to around £140 million for the UK population every year (this is likely to be significantly higher now). To date, there is little data concerning the mechanisms which underlie the sex-differences observed in patients with autoimmune disease.

In terms of animal benefit, by considering sex as an important biological variable we hope to increase long-term reproducibility of data by increasing the reporting of the sex used in animal experiments. This will lead to a long-term reduction in the number of animals used for experiment medicine. It may also lead to refined procedures where male and female mice are treated differently during experiments, as they are likely to benefit from different humane end-points depending on sex.

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

We will maximise the outputs from this work by communicating the research locally in departmental meetings and to the wider scientific community at national and international meetings. Once completed, this work will be published, open access, in journals with the highest possible impact. We will also publish negative findings in open content journals such as the Public Library Of Science (PLOS). At the Centre for Adolescent Rheumatology, there is also a thriving patient participation, involvement and engagement (PPIE) community, which will be used to disseminate and discuss ongoing findings with patients and their carers, to make sure that the animal research undertaken is relevant to patients.

Species and numbers of animals expected to be used

- Mice: 8500

Predicted harms

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

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

We will use mice in our experiments as these animals have well characterized immune systems that share many similarities to humans. Research with mice has been fundamental to the development of many drugs that are used to treat immune system related diseases. We are using adult and juvenile mice to allow us to compare how the immune system changes over age in both males and females.

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

An animal used in this project license will typically be given an inflammatory stimulus by injection to induce the development of a rheumatological disease such as arthritis or lupus. The experiments will last approximately 2-3 weeks. However, some models require us to keep the animals alive for longer (max 6 months).

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

As we are inducing inflammation and the development of autoimmune disease, we expect mice to experience some pain and swelling at the affected site. Depending on the model induced, we expect the pain to last between 1-3 weeks. In some mice, pain will be limited as we will be testing experimental substances that may suppress disease development.

Expected severity categories and the 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% of the animals on this project license.

Mild: - 50% of the animals on this project license.

Moderate: - 35% of the animals on this project license.

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?

Autoimmune diseases develop as a result of a the complex interaction between multiple organ systems and multiple immune system cell types. Thus, unfortunately, at present, we are unable to model autoimmune disease using isolated cells from humans or healthy animals, or cell-lines in a test tube.

The data and results obtained from experiments where we look at the kidneys, liver, spleen, blood, lymph nodes and joints of animals with disease are more representative of human disease. Kidneys will be taken from mice with lupus; joints will be taken from arthritic mice; liver, spleen, blood and lymph nodes will be taken from every mouse to analyse the immune response. Where possible we use cell lines or samples taken from humans instead.

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

We considered using human samples and cell lines.

Why were they not suitable?

Where possible we will use human samples. However, they are not suitable for the majority of this work as we can only access peripheral blood samples from human patients due to ethical limitations.

Autoimmune diseases are multi-organ diseases that affect many different sites and to understand why there are differences in susceptibility between men and women, we need to analyse the tissues that the disease affects such as kidney's and joints. It is also important to understand how diseased tissues 'talk' to healthy tissues and whether cells move between sites. This is also true of cell-lines which do not recapitulate the microenvironment of complex tissue anatomy. In addition, many cell lines do not state the sex of animal/human they are derived from which means they are not suitable for our study.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experiments and pilot data. The differences between the sexes were subtle and therefore a larger amount of mice are needed to obtain statistically significant results. However, as we are comparing sexes within every experiment, we have the ability to use experimental designs that reduce the amount of animal needed to get statistically significant results. As well as input from in-house statisticians, the NC3Rs experimental design tool will also be used.

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

Careful consideration is given to calculate the minimum number of mice needed to give statistically significant results. The size of experimental groups has been decided after consultation with the statistician at our institute and we will continue to consult with statisticians for optimization of the experiments throughout the life of 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?

We will take many steps to reduce the number of animals used. This includes purchasing wild-type animals from reputable commercial sources (reducing the numbers of mice needed to maintain a colony). We will also share tissues with other researchers in the department where possible. In addition, where we have new programme of work (e.g. cross hormone treatment in mice), we will start by carrying out pilot experiments to refine our protocols. We will then carry out power calculations prior to embarking on the full programme of 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.

Experimental procedures carried out on our mice can be broadly divided into two categories: inducible- models of autoimmune disease and spontaneous models of autoimmune disease. Mice used under these protocols will experience a moderate level of severity at most, and many steps are in place to prevent prolonged suffering or the development of a high level of severity. As the models we are using reflect human autoimmune diseases the adverse effects will include some inflammation, some weight loss, and well as some pain at inflamed sites.

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

Many autoimmune mouse models are only inducible in adult animals and take some days/weeks to develop. Unfortunately, this precludes the use of immature life stage animals and 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 use mice in our experiments as these animals have well characterized immune systems that share many similarities to humans. Research with mice has been fundamental to the development of many drugs that are used to treat immune system related diseases. In our research we will start by using inducible models of disease that result in short-term, highly reproducible disease with almost >95% incidence. The reproducibility and reliability of the mice getting disease will allow us to use fewer mice. Following identification of biological processes that may be involved in disease development, we will look at these pathways in spontaneous mouse models of autoimmunity that are more translationally relevant to human autoimmune disease, which is also spontaneous. In general, to minimise welfare harm, we inspect the mice daily and administer pain relief where possible. We will also use the most refined inoculation route and as much as possible use agents that illicit and immune response that are less inflammatory. We will also where possible use fewer toxic compounds to achieve the same goal.

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

We will use animal welfare scoring sheets, which were designed using advice from the following website:

https://www.humane-endpoints.info/en/score-sheets

We have also designed maximum sampling sheets for all researchers working on the PPL, using advice from the following website:

https://nc3rs.org.uk/blood-sampling-mouse

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

We will consult our NC3R regional programme specialist and check the NC3Rs website regularly. We will also attend courses through the university that update us on these regulations. Updates will be disseminated round the team working on the project license and in the case of big advances/changes to 3Rs recommendation we will have a lab meeting or short seminar to discuss in more detail.

27. Tumours - Therapy, Diagnosis and Models

Project duration

5 years 0 months

Project purpose

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

Key words

cancer, therapy, drug delivery, Nanomedicine, targeting

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 overall aim of this project is to allow evaluation of promising diagnostic and therapeutic approaches to cancer such as anti-cancer drugs or nanomedicinesand to identify relevant animal models or suitable in vitro replacements that provide predictive data for the clinical translation.

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

Why is it important to undertake this work?

Scientific and technological advances mean promising compounds and drug delivery technologies are being developed in the laboratory but to accelerate the transition of these into the clinic it is important to select only the most promising candidates for further clinical development.

The new molecules and delivery systems or nanomedicines are typically first tested and developed in cell-based assays. However, cancer cells growing in isolation in the

laboratory only replicated limited aspects of how drugs/delivery sytems are going to behave in actual tumours or inside the body. It therefore becomes necessary to test the most promising candidate systems in animals to ensure that they are efficacious and safe before they undergo further clinical development. Where we develop novel in vitro replacement models they will require validation in animals models to understand whether they are likely to be predictive of the clinical performance of such therapies. This will accelerate and simplify pre-clinical development and is well aligned with the objectives of the 3Rs.

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

The outputs from the project will include specific information about the effiacy and safety of novel therapies and diagnostics against a range of cancers; furthermore, they will bring improved mechanistic understanding of the underlying causes for this performance, and should allow identification of such factors which could be testable in vitro models and then allow pre-clinical in vivo validation of such models.

Examples would include our work on cancer gene therapy, anti-cancer peptide pill, improved therapy of Glioblastoma Multiforme (GBM) (as we have published over the last period) or ongoing work which includes improved therapy of child hood leukaemia, replacement of intravenous paclitaxel with oral/tablet formulations, targeting of breast cancers.

This information will further translate into outputs for dissemination (e.g. publications) and candidate interventions for clinical evaluation.

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

The immediate benefit will be to inform the scientific work of our group and our collaborators. In the near term (2-5 years) this will also inform the work of others working in this area in terms of knowledge and hypotheses for further development, but also improved or simplified models that may allow more rapid in vitro evaluation and reduction of downstream in vivo testing requirements.

In the longer term (5 - 15 years), our work will potentially create economic benefit linked to the development of novel interventions and ultimately to potential patient benefit with improved and safer cancer therapies and diagnostics if these clinical programs prove successful.

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

We will work with other scientists and engineers to make sure that we fully understand what these tiny particles are capable of as medicines. We will collaborate with scientist from other disciplines and consult with clinical colleagues regularly to focus the research on the most pressing clinical needs.

Where candidate interventions have been identified we will also consult other stakeholders e.g. patient groups.

We continue to facilitate translation of our efforts by identifying and supporting efforts to support clinical development via a commercial or non-commercial routes, as appropriate.

We will publish and disseminate all of our work regardless of whether it was successful or not in order to inform other scientists, in order to avoid duplication of in vivo work and to facilitate the development of appropriate replacements to in vivo models.

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.

The mouse is the lowest mammal to support the study of solid tumours in an organism. Cell culture experiments currently to not replicate the complexity of tumour development and the challenges of delivering therapeutics or diagnostics to tumours.

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

Tumour cells will be injected into the flank of animals so that they develop a small tumour under their skin over the next couple of weeks. Novel treatments previously tested on cells will then be given to the animals to evaluate whether they are likely to offer benefits in terms of future cancer therapy.

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

The growth of the tumour does not lead to apparent suffering. Similarly, the novel therapies and diagnostic interventions are not expected to be harmful. The procedures these animals will undergo are similar to those a human cancer patient would experience. This could therefore include adverse effects such as temporary unwellness or weight loss; animals having to undergo procedures will potentially cause temporary stress.

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

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

Around 90% of animals will fall into the moderate severity category. Other animals are expected to experience mild harms only.

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

- Killed

Replacement

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

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

Cancer cells grown in isolation are a very limited model for tumours which develop (and need to be treated) in an animal or human.

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

Cancer cells grown in vitro are used routinely before more promising approaches are evaluated in animals.

Why were they not suitable?

It is currently not possible to fully replicate the complex interactions of different cell types and 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?

For many of the experiments we have decades of experience in terms of typical animal numbers required. Where this is not known, pilot experiments allow us to carry out power calculations to estimate the minimal required animal numbers.

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

We are developing novel ways to allow multiple read-outs to be obtained from one experiment. Where desired effect sizes are known power calculation tools will be used (e.g. InVivoStat).

Tumorigenicity studies will be fully documented to gain profiles of the tumours to reduce repeats of pilot growth studies. Use literature and seek other establishments that have data on tumour models to know how best to grow tumours. Look at needle size: cell size to ensure the most appropriate needle is being used and doesn’t hinder the growth prospects of tumours.

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

Where there are significant unknowns in terms of experimental design or effects of novel interventions, we routinely use pilot experiments before planning pivotal 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.

Novel interventions will be first evaluated in normal mice without tumours to minimise suffering. Where tumours are essential for the evaluation, we typically work small tumours. Where interventions are likely to cause pain and suffering monitoring and analgesic interventions will further reduce suffering.

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

The mouse is the lowest mammal to allow replication of the most relevant elements of the tumour/body interactions. Aspects such as mammalian circulatory system, cells with macrophage activity (reticuloendothelial system), blood protein components, tumour stroma among other aspects are known to modulate nanoparticle biodistribution and would not allow meaningful results to be produced in lower species such as amphibians or nematodes.

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

The animals will be closely monitored after any intervention and where surgical procedures are required, they routinely will have the necessary post-operative care and pain management. Humane endpoints have been defined to prevent unnecessary suffering and where animals do show clinical signs these are recorded and suffering and distress assessed in a cumulative fashion using the Distress Score Sheet.

Monitoring will be adaptive, i.e. the frequency of monitoring will be adapted based on the nature of the procedure and the risk for suffering associated with it in the specific context of each experiment; for example, when animals are inoculated with tumour cells during the tumour development initial frequency for slow growing tumour models or tumour models in the perivascular phase will be lower than for more rapid growing tumours or tumours for which we have less experience with. This could mean animals will for example be clinically inspected daily in those situations.

Similarly, when animals are dosed with novel compounds e.g. in dose finding pilot experiments initially continuous and then more frequent monitoring may be required while for routine dosing with known drugs less regular monitoring may be sufficient.

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

All work will follow the guidelines published by Workman et al (2010, Br J Cancer, 102, 155).

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

Subscription to NC3R newsletter and alerts as well as CRACK-IT website; in addition to this academic journals that cover novel methods such as organ-on-a-chip etc.

28. The role of glia in nerve regeneration and cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, nerves, regeneration, glia, microenvironment

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The work in this proposal addresses how glial cells become cancer cells. The glial cells are found in the nervous system and are key ifor maintaining and repairing nerve cells. The development of tumours (tumourigenesis) appears to mimic the process that takes place in glial cells and the associated inflammatory and regenerative response following an injury to the nerve.. We therefore want to understand the nerve regeneration process to help us to understand tumour development and other diseases of nervous tissue. Moreover, these regenerative processes appear to mimic the growth of nerves into growing tumours, which appear to be important for both tumour growth and tumour spread. We aim to understand these processes using mouse models of tumour 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?

We study the mammalian peripheral nervous system to understand the establishment of a stable tissue, how a tissue can regenerate in the adult following an injury and how these processes may affect tumourigenesis. There is increasing evidence that the supply of nerves (innervation) is an important aspect of all solid tumour formation and is a

mechanism by which many tumours spread- a process known as perineural invasion (PNI). It is important to undertake this work to understand these processes, so we can improve nerve regeneration following injury and stop aberrant regeneration that can result in pain. Moreover, we would hope our findings will lead to new approaches for cancer preventention and treatment.

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

The primary potential benefit relates to new knowledge about the mechanisms underlying regeneration of the peripheral nervous system, the development of tumours in this tissue and the unappreciated importance of innervation and PNI in the initiation and spread of many tumour types. Additional benefits will include increased understanding of the blood nerve barrier in health and disease. This understanding could lead to new approaches for the treatments of diseases such as neuropathies, cancer, diabetis and neurptoxicity. The findings will be published in academic journals and are likely to be of great interest to pre-clinical and clinical scientists with interests in nerve injury, neuropathies and cancer. The second potential benefit relates to the possibility of the identification of new molecular targets and/ or devices, which may be of benefit for the treatment of these diseases. Evidence for this approach is the interest of clinicians and interactions with therapeutic companies based on our previous findings.

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

In the short term, the scientific field will benefit from an increased knowledge of nerve function, how nerves can be damaged and regenerate, the role of nerves in cancer and the blood nerve barrier. In the longer term, we would expect benefits to human disease. These include the development of new devices and protocols to improve nerve repair and to inhibit aberrant nerve regeneration. It may also

lead to new strategies to deliver drugs to the nervous system. In this regard, we are already in a collaboration with a pharmaceutical company in an attempt to increase the delivery of therapeutics into peripheral nerves. Other longer term benefits may include new approaches to prevent and treat cancer and to develop new approaches to protect patients from drugs that damage nerves as a side-effect, such as chemotherapy, which is a major limiting factor for chemotherapeutics.

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

We will maximise the outputs of this work through specific strategies.

To disseminate our research and discuss the results (including unsuccessful approaches) with scientists who are world-renowned experts in the relevant fields (These include experts in neuroscience, cancer and tissue regeneration), we will present our research findings in national and international conferences on an annual basis.

The results of our research will be published in high-profile scientific journals that normally reach the wider scientific community. Publications will be open access.

The techniques developed in our laboratories will be highlighted and shared with our collaborators and with other researchers during discussions in informal meetings and in the course of national and international conferences and publications. Advanced skills workshop run locally and we will are part of these communities aimed at sharing expertise in our Institute.

Species and numbers of animals expected to be used

- Mice: maximum of 4900 per year
- Rats: maximum of 1000 per year

Predicted harms

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

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

While we have made great attempts to develop in vitro (not in live animals) culture models, it is clear that it is currently impossible to fully model the behaviour of nerves or glial cells in vitro. This is particularly the case for studying the role of homeostasis (maintenance), regeneration and tumor formation of the complex peripheral nervous systm. We will therefore perform many of our studies using mouse models and in some cases cells derived from rats , although we will use the in vitro models when appropriate.

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

Many mice and rats will be sacrificed to provide tissue to produce cells or samples.

Some mice and rats will undergo peripheral nerve injury and will be injected with substances to analyse the process or treat the process. These mice may be kept for several months.

Some mice and rats will be injected with substances to analyse tumour formation or prevent tumour formation. These mice may be kept for several months.

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

Most of the experiments described in this application require the use of genetically-modified mice that express forms of genes that are mostly indistinguishable from any other mouse. . For a majority of the experiments, the animals will be bred to produce cells or tissue for in vitro experiments.

Some of the experiments will involve manipulation of genes in vivo (in living animals) that will result in tumour formation or nerve damage . Other experiments involve injections of tumour cells to assess tumorigenic development and spread. These animals will be monitored regularly and any animals showing signs of distress will be humanely culled. Based on previous work, the likelihood of distress in the animals is considered low and the

severity level is judged as moderate. Animals will not be allowed to develop tumours greater than 1500mm3 and the tumour mass will not exceed 10% of bodyweight.

Some experiments require the damage of a nerve in mice/rats. These experiments will be carried out under general anaesthesia. The recovering animal will be medicated for pain and closely monitored. Based on previous work the suffering is minimal as the animals are able to move around and feed normally. In the unlikely circumstance that any animals show signs of infection or discomfort, they will be humanely culled.

Some experiments involve in vivo imaging of cell migration. These animals will be anaesthetised and will be culled at the end of 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)?

Mild

- 47% of all mice will be in the mild category
- 33% of all rats will be in the mild category

Moderate

- 53% of all mice will be in the moderate category
- 67% of all rats will be in the moderate category

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

- Killed

Replacement

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

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

Complex processes such as nerve regeneration and tumorigenesis and tumor innervation cannot be satisfactory modelled in vitro. The role of the microenvironment in these processes is crucial and still widely unexplored, thus impossible to reproduce in in vitro conditions. Therefore it is necessary to use animal models.

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

We work in vitro with primary Schwann cells and can recapitulate some of the processes by which Schwann cells interact with other cell types. Moreover, we have in vitro assays for tumourigenesis. We have used both model systems to identify important signalling molecules involved in these processes and will continue to do so.

Why were they not suitable?

Many effects that we, and others, can observe and measure in vivo are not reproduced in vitrot. In particular, the role of the immune system and how cells behave in complex tissue environments cannot be reproduced in vitro. Therefore, it is always critical to test our models in animals.

Reduction

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

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

The proposed experimental designs and methods of analysis have been discussed with our experts in statistics to maximise the information obtained from the minimum resource. Randomisation and blinding methods will be used when appropriate.

Our extensive use of in-vitro methods limits the number of animals required for the in-vivo stage.

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

The proposed experimental designs and methods of analysis have been discussed with our experts in statistics. Where relevant, factorial experimental designs will be used to maximise the information obtained from the minimum resource. For most of the quantitative experiments, sample sizes may be set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 25%. The number of mice per treatment group will be calculated to provide statistical significance.

Our extensive use of in-vitro methods limits the number of animals required for the in-vivo stage. To minimise animal usage, prevent the unnecessary production of animals showing adverse effect and to ensure that animal breeding is inextricably linked to research requirement, the project licence holder will:

Ensure high standards of animal care, welfare and utilise the most appropriate breeding methods.

Ensure that colony sizes are monitored and adjusted within a formal forecasting system to meet the requirements of the research programme(s). Transgenic mouse lines that are not expected to be used in the next 6 months will be suppressed and embryos will be cryopreserved for future use

Ensure that breeding colonies are always kept to their minimum size so as not to over produce.

Ensure that Personal Licensees working on this project are appropriately trained and suitably competent to enable a high success rate to be achieved and thus minimise the number of animals used.

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

Additionally, to what is explained above, 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, we expect that 6-8 animals per treatment group should be sufficient to obtain the required results. For the tumorigenesis studies more mice per treatment group may be required, if tumour initiation does not occur in all animals of one treatment 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.

We will use mouse and rats for our studies. Generally, the anatomy, physiology and genetics of the mouse and rat are similar to humans, and thus they represent a powerful system to model human biology and disease. Rats and mice are the animals in which nerve structure and regeneration have been most studied and mice are the in vivo model of choice for tumorigenesis studies. The mouse is the only mammalian species for which transgenic techniques are widely available and is therefore the model of choice for genetic studies. It is widely accepted that mice NF1 tumour models are a good model for the human disease. Moreover, the nerve regeneration process is similar in rodents and humans.

For our nerve injury studies, only one nerve is injured and the animals are able to move freely with such damage. For our tumorigenesis studies, the animals are sacrificed prior to the animals suffering any measurable distress.

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

We are studying processes that need to occur in a living, adult animal often taking place over days- months, therefore terminal anaesthetised animals are not suitable. Less sentient animals do not show similar behaviour or there are not suitable genetically modified animals.

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

Each protocol used is carefully designed to ensure physiological significance and to minimise suffering and where appropriate we are developing relevant in vitro models.

The mice and rats are kept in purpose-built accommodation. The animals are mostly kept together for companionship and are given materials to encourage their natural behaviour. The animals are bred and cared for by a dedicated team of highly-skilled technicians. Animals are closely monitored for signs of ill-health or distress. The technicians have the expert assistance of two "named persons", the named Person Responsible for Animal Care and Welfare and the Named Veterinary Surgeon.

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

Some experiments require the damage of a nerve in mice/rats or injection of cells of substances into nerves, neck, mammary fat pad or the pancreas. These experiments will be carried out under general anaesthesia. The recovering animal will be medicated for pain and closely monitored. Based on previous work the suffering is minimal as the animals are able to move around and feed normally. Any animals showing signs of infection or distress will be humanely culled.

Some experiments require testing for the ability to form tumours in vivo. Animals will not be allowed to develop tumours greater than 1500mm3 and the tumour mass will not exceed 10% of bodyweight. Any animals showing signs of infection or discomfort will be humanely culled.

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

We are in close contact with the animal housing staff and exchange news and advances in the field of animal welfare. The website of the National Centre for the replacement refinement & reduction of Animals in Research (nc3rs.org.uk) is a great additional resource to check for advances and updates on this topic.

29. Development of non-invasive imaging markers for understanding cancer biology and detection of treatment response

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
- 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, therapy, biomarkers, microenvironment

Animal types	Life stages
Mice	juvenile, adult
Rats	juvenile, adult

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to develop in vivo imaging markers of tumour metabolism, hypoxia, blood flow and structure which can then be used to evaluate treatment response.

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

Why is it important to undertake this work?

Despite the use of aggressive combinations of surgery, radiation therapy and chemotherapy, cancer remains incurable in most cases. This is due to a lack of effective treatments as well as an absence of sensitive non-invasive imaging methods that can accurately detect early treatment response so that alternative treatment can be performed before it is too late. While newer drugs and treatment modalities are entering clinical trials, treatment efficacy is generally evaluated in the clinic using a decrease in tumour volume. Unfortunately, a change in tumour volume occurs relatively late after starting therapy and thus, may not be able to detect early resistance to therapy that would warrant a change in treatment. It is therefore, important to develop accurate and sensitive imaging methods that can detect and assess early response to therapy. Using multi-modal imaging technologies, we aim to develop imaging markers that would not only be complementary to each other, but provide sensitive and specific markers for the underlying biology of the tumour. Such markers would be more efficacious than simple changes in tumour volume. In addition, as many of the proposed imaging technologies such as MRI and PET are routinely used in the clinic, these markers could potentially be readily translated to the clinic to aid in cancer diagnosis and monitoring of treatment response.

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

Unlike traditional methods of evaluating treatment response in cancer such as biomarker assays from blood samples that lack spatial information or post-mortem pathology analyses, in vivo imaging methods provide non-invasive assessment of the tumour in three dimensions, providing a comprehensive picture of tumour heterogeneity. As such imaging can be performed non-invasively under anaesthesia, the same animal can be imaged repeatedly, substantially reducing the number of animals required. Similarly, the same animal can be used as its own control reducing the biological variability, which further reduces the number of animals needed in order to achieve statistical significance.

The proposed programme of work will exploit the complementarity of imaging modalities in probing the basic mechanisms of cancer growth and metastasis. Further refinement of existing methods as well as development of newer quantitative imaging biomarkers will be used as surrogates of early treatment response for a range of therapeutic interventions including conventional chemotherapies, targeted drugs, radiation therapy, and kinase inhibitors as well as cell therapies. The data are typically comprised of images or spectra from which quantitative parameters of tumour perfusion, hypoxia, metabolism and heterogeneity can be derived.

Research outputs will include:

1: The research will provide insight on the ability of preclinical imaging to detect and characterise tumours, and to detect their early response to chemo- or radiotherapies. Drug and contrast agent toxicity and efficacy will also be assessed. Novel imaging methodology will be assessed and optimized for in vivo tumour assessment.

2: Preclinical in vivo imaging will allow reduction in the number of animals by reducing the reliance on post-mortem biomarker assays and cohort studies. Imaging methods are also minimally invasive reducing the burden of suffering on the animal refinement. Therefore,

developing these methods creates an avenue into cancer research with a substantially reduced burden placed on the animals.

3: Most of the imaging methods proposed are functionally equivalent to human clinical practice. The outcomes of the research therefore have a direct and obvious route to clinical translation and potential to influence the standard of clinical care.

4: The results will be published in peer reviewed journals to disseminate the findings of this work so that the research community can use and build upon the scientific findings. We will also share the potential benefits of preclinical in vivo imaging with the research community at large. Any novel methods developed can then be implemented at other sites improving best practice.

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

Short term:

This project aims to develop a series of imaging markers for treatment response of tumours and to determine whether they can detect treatment response earlier than traditional measures of tumour volume change. This work will directly benefit basic researchers working on cancer biology and treatment, especially regarding treatment response, the mechanisms of response and timescale over which that response occurs.

We also aim to develop novel/improved in vivo imaging methodologies. This work benefits preclinical imaging research as it demonstrates new techniques and ways to interrogate in vivo biological systems, metabolism, and microstructure. This research has general application well beyond cancer as in vivo imaging can be applied to image any tissue (e.g. liver, brain).

The project will also provide insight into tumour response to drug or radiotherapy. It should reveal the timescales of the treatment response in a variety of tumour models providing a solid starting point for further imaging biomarker research and validation.

Medium term:

Clinical research into imaging biomarkers of tumour response to treatment will benefit from the output of this project. As the imaging methods proposed in this project are largely based on technologies routinely used in the clinic, this project will provide a starting point for investigating the potential for a clinical imaging biomarker of early treatment response. The project should also provide a detailed investigation of the biochemical, genetic, and structural changes underlying observed imaging responses aiding in their clinical interpretation.

This project will provide tools to investigate the response of preclinical cancer models to potential new clinical treatments, particularly with regards to blood supply/neovascularisation, hypoxia and drug response. This will benefit preclinical cancer research.

There is the potential to investigate whether such observations as are made in preclinical tumour models can be replicated in a human clinical context. The fact that preclinical and

clinical in vivo imaging is largely based on similar fundamental technology enables relatively straightforward clinical translation of methods. This will benefit researchers working on understanding human tumour biology.

Longer term:

In the much longer term, this project aims to lay the groundwork for potential validation of clinical imaging biomarkers for treatment response in cancer. Without the understanding of the fundamental biology underlying imaging markers of cancer, it is difficult to interpret imaging biomarker results. Such biomarkers would enable clinicians to change treatment regime early in the treatment time course when that treatment proves ineffective/inadequate. Such biomarkers would benefit both clinicians by providing new tools for treatment/diagnosis/stratification of patients and also patients who would receive a more personalized healthcare better adapted to their unique pathologies.

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

Our work is highly collaborative in nature and our ongoing collaborations have resulted in multiple where we have primarily assessed changes in spectroscopic markers of treatment response. Our ongoing collaborations focus on development of a novel MRI marker to measure tissue conductivity as a contrast mechanism to understand the tumour microenvironment. The results of the imaging experiments, including negative experiments, will be published in peer reviewed journals that will aid other researchers.

Our proposed work on PDX models will aid in maintaining the clinical relevance of our work and in driving clinical translation of imaging studies. We have strong collaborations with treating oncologists and with oncology surgeons that helps us focusing the direction of our research such that it remains clinically relevant. This aids in maximising the translational potential of our work and its relevance on clinically relevant aspects of tumour biology.

Species and numbers of animals expected to be used

- Mice: 1050
- Rats: 1050

Predicted harms

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

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

Rodents are the most commonly used model animals in cancer research because of the marked similarity of their genome to the human genome, relative ease in the development of tumours, cost and reproducibility. A substantial body of published work exists on which further work can be based, along with available and characterized rodent tumour cell lines. We have chosen to use either juvenile or adult animals as the tumour take rate is much

higher in these age groups and because most tumours in humans occur during these stages of life.

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

Most animals will typically undergo either injections of tumour cells or surgical procedures for implantation of tumour cells under anaesthesia. Depending on the tumour type (growth pattern), it may take anywhere between a few days to several weeks (8-10, in rare occasions) for the tumours to grow. Longitudinal imaging studies (under anaesthesia) will be performed to evaluate tumour growth, physiology, metabolism or response to treatment with therapeutic drugs. Imaging studies may involve injection of contrast agents and therapeutic studies may involve administration of drugs. Animals may undergo multiple imaging studies to evaluate changes in tumour metabolism or organ function in order to develop novel imaging methods as markers of assessing treatment response, particularly early treatment response.

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

Surgical procedures are likely to cause pain. However, these procedures are performed under anaesthesia and the animals recover normally in most cases. Development of tumours may cause discomfort, pain, or other clinical signs and some drugs may induce toxicity. Most of these effects are transient in nature and will be closely monitored. If clinical signs of suffering are present, the animal will be either treated with analgesia or euthanized as necessary using the scoring sheets (Tables 1-5) .

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)?

In naive animals, we expect the severity to be from mild (most) to moderate (about 5 - 10%). In tumour bearing animals, we expect the severity to be mild in subcutaneously injected tumours. In a limited number of cases (5-10%) invasion of subcutaneous tumours into underlying tissue may occur. We expect the severity to be moderate in most orthotopic and metastatic cancer models.

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 tumour/host interaction is critical for the support and survival of the tumour, recapitulating the clinical case requires the study of an orthotopic tumour. This system cannot be recapitulated using a different model system. As such, a proper understanding of tumour growth and response assessment can only be performed on live animals. In addition, potential toxicity of the therapeutic agents to normal organs cannot be assessed in cell-based systems.

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

In vitro studies on cells and organoids can provide some information on the growth pattern or how effective is a novel drug in killing tumour cells. Similarly, use of the chick embryo CAM model has some potential to allow initial testing of novel drugs targeting, for example, angiogenesis. Unfortunately, due to the small size of the tumours grown in the CAM model, and the lack of interaction with host tissue and immune system, its application beyond these initial stages is limited.

Why were they not suitable?

In vitro cell studies or on organoids will not be able to tell us the effects of the tumour host environment (location, blood flow) and its interaction with immune cells of the body (T-cells, macrophages). Similarly, due to an absence of host vasculature these alternatives are unable to determine the role of blood flow in tumour hypoxia and in the delivery, and hence efficacy, of therapeutic drugs in the treatment of cancer. In addition, these alternatives do not mimic the clinical scenario and hence the imaging methods (on cells) would not be translatable to 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 number of animals was estimated that would be sufficient to distinguish tumour from normal healthy tissue when evaluating the relative effects/abilities of different imaging contrast agents and imaging methods. In addition, as we will evaluate several therapeutic drugs, both as monotherapy and combination therapy, we estimated the minimum number of animals needed to detect statistically significant differences between treatment and control animals. This is based on several published studies including studies using the novel choline kinase inhibitor JAS239 that we have previously used in work conducted under our existing Project Licence.

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

We have used the NC3Rs EDA tool for experimental design as well as consultation with the statistician, to determine the minimum number of animals needed to test each hypothesis under different experimental conditions. By using longitudinal studies, each animal can serve as its own control further reducing the number of animals required and avoiding the need for larger cohort studies. Similarly, the experiments are designed to provide multiple pieces of data from each cohort 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 pilot studies in a small cohort of animals to determine contrast agent biodistribution, optimisation of image contrast and sensitivity, or for evaluating drug toxicity while using therapeutic agents. This allows method development/optimization before implanting tumours in larger numbers of animals. This reduces the risk that technical optimisations may compromise the entire experimental cohort. The developed imaging contrast agents as well as imaging protocols will not only be useful for reducing the number of animals needed for drug studies in this project, but this information will be shared with other cancer researchers so that they do not need to repeat these studies. Since we are primarily interested in assessing cancerous tissue, we will share any other tissues/organs with other researchers at the end of our experiments.

Refinement

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

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

We will use rodent cancer models as they are well established and extensively used in preclinical research settings. The models have been chosen due to their suitability for the imaging technologies to be tested in the project and for being relevant to the human clinical conditions. The subcutaneous tumour models will cause the least suffering to the animals and will be used where possible. Where subcutaneous tumours provide an adequate scientific analogue of the orthotopic tumour, initial studies will be conducted in subcutaneous tumours where the suffering imposed upon the animals is minimized. Orthotopic models are needed to recapitulate the clinical human tumours and may cause some suffering to the animals; these protocols have been designed to minimize this suffering as much as possible.

Use of in vivo preclinical imaging to assess tumour physiology inflicts a minimum of suffering on the animals as the procedures are non-invasive and performed while the animal is under general anaesthesia. As alternative methods to assess tumour biology are invasive and destructive, preclinical imaging is a considerably better option in terms of

animal welfare. Where possible, animal handling and physiological maintenance while undergoing imaging is under continual development and improvement.

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

Less sentient models, including development of cancer models in the chicken embryo are currently being evaluated for some studies. Whilst these models are useful for some aspects of the research, such as comparing tumour growth pattern differences between different cancer cell lines, they do not fully recapitulate the human clinical condition. As the tumours are typically grown in the CAM membrane of the chicken egg, it is not possible to evaluate the host-tissue interaction and its impact on tumour survival or upon therapeutic delivery of drugs.

Since we are developing imaging methods to assess longitudinal changes in the tumour microenvironment and to assess treatment response in the same animal, it is not possible to test these methods in terminally anesthetised animals.

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

The NVS will be consulted and any recommendations for alleviating pain or improving pain management (e.g. analgesics) will be provided to the animals. Monitoring of vital signs during surgical procedures and during imaging is standard practice in our laboratory. In addition, we closely and frequently monitor animals in the post-surgical phase and after the tumours become visible on in vivo imaging. Where a procedure that is less invasive or incurs a lower burden of suffering on the animals (e.g. subcutaneous tumour model) proves to be adequate to answer a scientific question, it will be used in place of more invasive procedures. Optimization of imaging methods as part of this project also allows methods that are demonstrated to be unnecessary, or ineffective, to be omitted from further studies and for total imaging times to be reduced.

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

We follow the NC3R guidelines for imaging methods and tumour models to perform the studies.

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

Non-invasive imaging has been recognised as a key tool in 3Rs, especially for reducing the number of animals required for research. Since the same animal can be imaged several times thereby reducing the need to cull the animals at each time point, conventional cohort-based methods of assessing drug distribution or for assessing treatment response are not required.

In addition, we work with NVS and BSU and actively engage with NC3R to keep ourselves abreast of the advances in 3Rs and have implemented some of the refinement methods already in our existing project. We will continue to work with relevant authorities and will

effectively implement any advances in the 3Rs in relation to our imaging work and tumour models.

30. Individual health and population processes in wild ruminants.

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

Population, Ecology, Genetics, Immunology, Microbiome

Animal types	Life stages
Sheep	neonate, juvenile, adult, aged, pregnant
Red deer	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 causes and consequences of variation in the gut ecosystem; to determine the sources, mechanisms and impacts of maternal effects in the wild and to determine the mechanisms by which populations are changing under environmental change.

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

Why is it important to undertake this work?

Understanding how free-living populations work is critical to informing the management of over- abundant species and the conservation of endangered species. As our planet warms

and humans modify or destroy natural habitats, the pressures on wild animal populations are changing and intensifying. Our individual-based studies provide detailed insights into how animal populations respond to natural environmental conditions, allowing us to predict how they will respond to new environmental pressures and whether they can persist in the face of rapid environmental change.

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

Conservation of Soay sheep:

Data and tools to inform and improve deer management: Our research on red deer will benefit landowners and managers of deer populations across the UK. Our study population is the only closely monitored deer population in the region, and by improving our understanding of how and why deer numbers fluctuate naturally in response to environmental variation, we can help inform deer management strategies. For example, outcomes of our research would inform and allow managers to vary their culling strategies depending on annual climate conditions or local habitat conditions to provide a more refined and adaptive deer management. As calving date advances, we can also advise on the determination of open and close season dates.

Discoveries of fundamental scientific importance: Our work will shed important new light on how gut ecosystems, maternal effects and climate change shape the dynamics of unmanaged animal populations. Our findings will offer new understanding and shape thinking across a broad range of related scientific disciplines, including ecology, evolutionary biology, behaviour, space use, demography, microbiology, parasitology, immunology, and epidemiology. There are few, if any, other long-term field studies of vertebrates that have the requisite baseline data and sampling capacity to tackle these questions with the power and completeness offered by the sheep and red deer systems we work with. As well as providing new discoveries relating to our overall aim and individual objectives, we will use our discoveries to make computer models which will offer insights and opportunities for further study and investigation far beyond these two study systems.

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

The scientific research community (including animal behaviour, ecology, evolutionary biology, demography, microbiology, parasitology, immunology, epidemiology): The new discoveries provided by our research and from the new technologies and computer models we develop using our data and findings. Our findings will help provide important answers to open questions about the role of gut ecosystems, maternal effects, animal behaviour and climate change in driving variation in life histories and population dynamics in naturally regulated systems and provide new tools to more accurately predict the consequences of environmental change or disease outbreaks for wildlife and livestock.

Veterinarians and farmers (Soay sheep research): In the UK, gut worm infections cost the livestock farming industry an estimated £80 million annually, whilst gut protozoan infections result in an estimated 6-9% gross margin reduction. Resistance to available worming drugs is emerging rapidly and is a major current concern for the industry and

policy makers. The non-invasive monitoring methods we will develop to monitor gut parasite communities in Soay sheep can be applied in agriculture to monitor changes after drug treatment to better understand the consequences for different worm and protozoan species and improve targeting of treatment. Furthermore, the epidemiological models we will develop can help identify optimal strategies to reduce worming treatment and will offer a platform for the development of evolutionary models of worm drug resistance to help limit its spread.

Wildlife managers (red deer research): Our research on red deer will benefit landowner and managers of deer and other wildlife populations. Our study population is the only closely monitored deer population in the region, and by improving our understanding of how and why deer numbers fluctuate naturally in response to environmental variation, we can help inform deer management strategies.

Outcomes from our research will allow managers to vary their culling strategies depending on annual climate conditions or local habitat conditions to provide a more refined and adaptive programme of deer management. As calving date advances, we can also advise on the determination of open and close season dates.

Conservation: The conservation of the unique lineage of Soay sheep has intrinsic value to many members of the public, as well as conservationists, historians and farmers. Our research will enable us to monitor threats to the Soay sheep population's viability, for instance from climate change or novel infections, and work with the archipelago's owners to develop and implement mitigating actions. For example, our continuous screening of the bacterial, protozoan and worm diversity in the Soay sheep should identify any novel infectious or pathological agents that might accidentally be. More generally, we anticipate our work on gut ecosystems will have significant impacts for in- and ex-situ conservation management. We will be developing methods to monitor diet and gut community structure from faecal samples, and new techniques to analyse the data produced. Wildlife and conservation managers often need to know how gut health and community structure differs between wild and captive individuals (e.g. for reintroduction programs), and the nature and degree of parasite transmission to threatened wildlife species (e.g. from livestock). Our project can provide readily useable and transferable protocols, tutorials and support in all these areas, offering more affordable and flexible methods and allowing conservation organisations to reduce reliance on commercial kits or services. Not only will this save them money, but crucially it will increase capacity to perform monitoring in developing countries, where sample transfer is difficult for legislative or political reasons.

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

Scientific community: Through publication of our work in respected peer-reviewed journals in appropriate disciplines, and presentations of our work at meetings and conferences. Additionally, our work is publicised via our project websites and associated Twitter accounts.

Veterinarians and farmers (Soay sheep research): Our collaborators at other research institutes and universities have close contact with the veterinary and agricultural communities and we will work with them to communicate relevant findings via displays at

agricultural shows, presentations at meetings and conferences, and educational webpages, videos and animations.

Wildlife managers (red deer research): Our research can help management of red deer in the study area specifically, through communication with the area's owner, and nationally through communication with deer management organisations. We will also use our close contacts within the deer management community across the UK to disseminate findings and support their use in deer management practices and plans.

Conservation: We will continue to work closely with the owner and manager of the area and communicate any findings relevant to the conservation of Soay sheep to them and help develop and implement efforts to mitigate any threats we identify. We have also established links with conservation biologists and will work with them to communicate the methods we develop and discoveries we make about the gut ecosystems of Soay sheep and red deer to the conservation community.

Species and numbers of animals expected to be used

- Sheep: 1800
- Red deer: 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.

If we wish to understand the factors governing variation between individuals in natural populations and how they build up to population level processes like population dynamics and evolutionary change, then we have to study animals in the wild. Abundant diurnal mammals make excellent subjects for this work because they can be easily observed and they cannot disperse by flying or swimming - so it is possible to get very high quality information on breeding success and survival. In each study area we try to catch and mark all individuals as soon as possible after birth in order to be able to follow individual life histories from the start and for the rest of their lives.

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

Soay sheep:

Typically (for ~90% of sheep born in the study area), a sheep is caught as a neonate in the first few days of life and a blood sample, ear punch and morphometrics are obtained and identification tags applied and then it is released back to its mother. Each August around 50% of the animals resident in the study area are then caught in corral traps and blood samples and morphometrics are obtained. If an animals has not been caught before, ear punches are taken and identification tags applied. All animals are then released to the wild. A sheep that lives for several years may be caught every summer or not at all if it lives on the edge of the study area.

A small number (~35 per year) of untagged sheep, nearly all males that enter the study area to rut, are caught by darting and anaesthesia. A blood sample, ear punch and morphometrics are obtained and identification tags applied before reversal and release to wild. Unless such sheep become resident in the study area, these individuals are rarely caught again in their lives.

Red deer

Typically (for ~90% of deer born in the study area), a deer is caught as a neonate in the first few days of life during the lying out phase and a blood sample, ear punch and morphometrics are obtained and identification tags (and if female, expanding collar) applied and then it is settled back in the place where the mother is expecting it. Once caught, individuals are only very rarely caught again in their lives.

A small number of adults (up to ~20 per year) are caught by darting and anesthesia to allow sampling and marking of animals that have not been caught during early life or to allow for reapplication of lost identification marks before reversal and release to wild. We do not dart animals a second time.

Apart from these events all animals are monitored remotely throughout their lives until lost to death or follow up.

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

To minimise adverse effects during capture of young, the handling of neonates is kept to within 10 minutes and animals are observed closely after release to ensure they are successfully reunited with their mother.

To minimise adverse effects during anaesthesia, we avoid anesthetising animals that appear in poor body condition and we watch animals until they appear to be fully alert and mobile. We do not anaesthetise any individual repeatedly.

In the sheep, when we capture animals in corral traps, we monitor individuals closely while they are in the traps, prioritise the processing and release of any animals that appear in poor condition and observe animals closely post release

Expected severity categories and the 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 severities are expected to be mild.

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

- Set free
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 situ ecological, evolutionary and conservation studies such as ours cannot be replaced by studies of cells in Petri dishes or computer simulations, because we need to observe individuals over lifetimes as they face the variation thrown at them by the (unpredictable) natural environment.

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

We have considered using in silico models of individuals and communities based on existing empirical data collected from free-living animal populations, and we have considered undertaking a purely observational study which did not require sampling or capture of any sort from our deer and sheep populations.

Why were they not suitable?

There is only limited empirical data available from natural populations capable of informing in silico models of the ecological and evolutionary dynamics of such systems. This is particularly notable in regard to the questions of how gut communities influence the dynamics of host populations in the wild, where few if any suitable longitudinal studies exist currently. Without studies such as those we are proposing here, we cannot build well-informed in silico models that would allow us to understand and predict such dynamics. Purely observational studies, without capture and sampling of individuals, would also not provide the necessary information to address our scientific questions as they would not allow us to monitor individuals longitudinally through time or to obtain samples required to link genotype, physiology, gut ecosystems and fitness.

Reduction

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

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

The estimates are based on the number of unmarked individuals we have caught per year over the last five years of study as neonates or older individuals in each study system.

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

Note that these are not experimental studies in the sense of manipulations with treatments and controls, they are observational studies.

We study all the individuals living in specific study areas, because we wish to understand how events at the level of individuals build into the higher properties of the populations (e.g. population dynamics). The size of these study areas is set so as to be practically manageable for field data collection and to provide sufficient sample sizes for statistical analysis. Although it does not result in any reduction per se, we are extremely keen on sharing our data with other researchers for analyses of new questions, to maximise the use of the data collected across research questions and scientific disciplines.

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

We catch and process as many individuals living in our study areas as possible.

In the case of the sheep born in the study area, ideally this is once at birth and once each August thereafter. For rams born out with the study area which immigrate for the mating season, we aim for only one capture.

In the case of deer we capture once at birth or once as an adult by darting. Occasionally, we dart deer that have lost all their marks to replace the marks.

Refinement

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

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

Which animal models and methods 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 do not use animal models in the parlance of mouse research. We use wild sheep and deer. Our research relies on observation of what happens to individuals due to natural processes. It would be counter to our objectives to cause such suffering that the lives of animals were affected by capture.

Therefore, our protocols are designed to cause minimal suffering before the animal is released back into the wild. For example, we aim to capture, process and release a lamb or calf within 10 minutes.

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

We require to follow entire life histories so immature life stages or terminally anaesthetised animals would be inappropriate.

Species that are less sentient: There are a small number of long term, individual based studies around the world like ours and they are mostly on vertebrates (mostly birds and mammals). This is a reflection of the fact that it is impossible to collect such detailed information on, for example, individual insects in the wild.

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

We are always looking for ways that might improve our capture and processing for the animals. Whenever more refined approaches become available during the course of this study either through personal communication, publications or veterinary advice, we will investigate their use.

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

With our kinds of projects, the appropriate activity here is to analyse the data by the most powerful and appropriate statistical methods possible and to publish our code and data alongside our papers so the community can replicate our analyses. This is now widely encouraged by journals in our research area.

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

I will monitor all opportunities for talks and meetings on the subject and attend where I can and implement advances if they are appropriate.

31. Understanding the Factors that Regulate Wound Healing

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Wound Healing, Diabetes, Ageing, Skin, Senescence

Animal types	Life stages
Mice	adult, aged, neonate, juvenile, 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 overall aim of this project licence is to provide new insight into the processes that regulate normal wound repair and to specifically identify factors that lead to poor healing in aged and diabetic mice.

This will support development of future targeted therapies to promote healing in aged and diabetic 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?

Chronic, non-healing wounds (which include diabetic foot ulcers, venous ulcers and pressure sores) are a major problem in the elderly and diabetic. Poor wound healing causes extensive psychological and physical suffering to patients, often leading to severe amputations and loss of life. The number of people affected by chronic wounds is

increasing due to the ever expanding elderly and diabetic populations. It is predicted that by 2025 there will be 5.4 million people in the UK with diabetes, while one in five people living in the UK will be over the age of 65. It is therefore not surprising that non- healing wounds are also a huge financial burden, currently costing the NHS a staggering £5 billion each year to treat.

Unfortunately, many non-healing wounds persist for months to years, preventing patients from living normal lives. Yet poor healing remains an entirely underappreciated area. Current treatments are wholly inadequate due to incomplete understanding of the factors that cause delayed healing in the elderly and diabetic. The work outlined in this application is specifically designed to address this gap in knowledge and support the development of more effective treatments to promote the healing of human chronic wounds.

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

This work is expected to provide new insight into the factors regulating normal healing and will identify new therapeutic targets for delayed healing in ageing and diabetes. This is important as non-healing wounds cause substantial physical, social, and psychological effects to patients and are a huge economic burden to the NHS. However, there are currently no universally effective treatments for non- healing wounds, with many patients facing life-changing amputations or loss of life entirely.

Academic Benefits

The work outlined in this project licence will uncover new factors that are associated with normal healing and are altered in poor healing (aged and diabetic) wounds. A focus of the project will be understanding how ageing at the cell level contributes to the way that skin heals. Wound healing is a major research field with over 10,000 research articles published each year. Our research will be of interest to a wide range of scientists in fields spanning biomedical sciences, basic biology and chemistry. Publication of this work will additionally allow researchers in other biomedical fields to explore whether our newly identified targets also contribute to other diseases (e.g. cancers). We will publish big data sets (e.g. genetic data containing over 40,000 genes) that can be used by other researchers as a resource to identify targets for their own research purposes. This will reduce the need for others to repeat this work, allow other researchers to use less animals in their own research, and it will increase the likelihood of others obtaining useful and valid results.

We will combine our animal research data with our findings in human models, which will result in a number of high impact publications in field-relevant journals and dissemination at international conferences. This approach will maximise the likelihood that the outputs from this licence will lead to follow on funding and first-in-man clinical studies. In addition, we will have the opportunity to patent our work, both in identifying new targets and re-purposing drugs for wound healing.

Patient Benefits

We will identify new factors that contribute to poor healing and use drugs to target these factors to see if they improve wound repair. We will first validate their effects in our human cell and skin models in the laboratory, before confirming their efficacy in our aged and diabetic mouse models. If these drugs prove effective in promoting wound healing in aged and diabetic mice, this will provide the proof we require to move these drugs into clinical studies for patient benefit within 3-5 years. Overall, this work could result in new, effective and safer treatments for non-healing wounds in humans.

Finally, if we identify a range of targets (biomarkers) that are linked to poor wound healing, then these can be used by clinicians to predict whether patients will heal their wounds or not. This would improve patient care by allowing patients, with wounds predicted not to heal, to receive tailored early treatments before it is too late.

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

In the short-term (1-3 years) researchers linked to this project (and others in the field) will benefit through disseminating work at conferences, publishing scientific articles, patenting novel targets and re- purposed drugs, and receiving grant funding from pilot studies.

In the mid-term (3-5 years) identification and validation of therapeutic targets for poor healing will lead to setting up clinical trials that will begin to directly benefit patients. At this stage trials can also be set up to determine whether identified targets (biomarkers) can be used to predict whether patient wounds will heal.

In the long-term (5-10 years) we would expect to see wider patient benefit if clinical trials prove effective. This will include:

The clinical implementation of a new and effective therapy for the treatment of non-healing wounds.

The ability to classify patients based on biomarker identification, which will ensure patients receive the care and treatment required to improve healing outcome.

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

Outputs of this project will be maximised as follows:

We will meet often to discuss findings within our group, at seminars and with collaborators, including our clinical partners to determine how the research can be used for patient benefit.

Research findings and publications will be disseminated to the general public through outreach events, patient support groups and via our communications team.

We will aim to publish our findings, even if they do not show a positive effect, to prevent others from repeating the work unnecessarily.

We will publish our big data sets to provide a substantial resource (40,000 genes plus) for the research community to use in their work. This will significantly enhance the field by

allowing other researchers to identify whether their targets of interest are linked to poor healing.

Species and numbers of animals expected to be used

- Mice: 2080

Predicted harms

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

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

Wound healing is a complex process that involves multiple cell types that behave in different ways throughout the course of repair. The wound healing process has evolved over millions of years to allow us to heal injuries quickly and efficiently. Lower organisms (e.g. flies and frogs) lack some of this complexity and have very different skin structures to humans. The most widely used models for human wound healing are mice, rats and pigs. These animals go through the same wound healing stages as humans and have very similar skin structure and function. For our work to translate to the clinic to benefit patients, we need to use models that better mimic the human wound healing response.

We use mice because they are the least sentient of the appropriate models and can be easily genetically altered to explore the influence of certain genes to wound repair. There are also widely published and validated mouse models of ageing and diabetes. As our major aim is to understand the factors that drive poor healing in the elderly and diabetic, these models will give us important information about ageing and diabetes that can be used to improve poor healing in humans. We also require adult mice because the immune system and skin of young mice is not yet fully developed.

Wound healing in underdeveloped skin is completely different to what we see in adults and will therefore not give us useful information that can be translated to the clinic.

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

An animal will either be bought in from a registered supplier or bred in-house for use in experiments. When the mouse reaches adulthood, it will be given general anaesthesia to fall asleep. The mouse will be placed on a heated pad to keep it warm, and the back of the mouse will then be shaved with clippers and cleaned. Two small skin cuts will be made on the back of the mouse while it is asleep, and pain relief will be given via an injection to prevent any short-term pain when the mouse wakes up. While the mouse is still anaesthetised, we may also add treatments to the wounds. The mouse will then be moved to a fresh cage with fresh bedding, food and water and will be placed in a heated, ventilated cabinet to recover. The mouse would only be under general anaesthetic for a few minutes and would wake up within one or two minutes of the procedure. The mouse would then typically be put under brief general anaesthetic every few days to allow us to take digital images of the wounds as they heal. The mouse would not undergo any other procedures.

Imaging would only take a minute and the mouse would only be asleep long enough to take a photograph of the wounds. At the end of the experiment, the mouse will be culled painlessly, and the wounds will be collected for laboratory analysis.

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

There are no expected adverse effects from the procedures performed other than potential mild and transient pain. All animals are expected to make a rapid recovery from the anaesthetic and should only experience transient pain from their small skin wounds. They will be given pain relief at the time of surgery.

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

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

Genetically altered mice bred under the breeding protocol are not expected to experience any harmful phenotype (100%). The wounding procedure itself is not expected to cause more than mild and transient pain (90% of animals). However, the diabetic mice we will use will gain weight and develop diabetes, which in itself increases the severity of their experience to moderate. Up to 20% of the mice we will use in wounding procedures will be diabetic. As diabetes is a progressive disease, we will also only use diabetic mice up until a certain age, to limit the severity of their experience.

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

- Killed

Replacement

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

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

The wound healing process has evolved over millions of years to rapidly repair damage to injured tissues. It is an immensely complex process involving interaction between circulating immune cells, local tissue cells, signalling molecules and the tissue matrix that surrounds cells. Skin is also a highly complex tissue which includes blood vessels, nerves, hair follicles, sweat glands and sebaceous glands. There are no current in vitro (cell) or ex vivo (whole tissue) models that come close to fully replicating the complexity of the wound healing process.

A number of animal models are available that heal wounds in a broadly similar manner to humans. The main models used are pigs and rodents, but in this study we will use the least sentient, informative model (mice). There is extensive published literature demonstrating that:

Mouse skin structure broadly resembles human skin structure.

The main processes that are involved in tissue repair (inflammation, proliferation and matrix remodelling) are all found in mouse wound healing.

In addition, this project will explore the causes of delayed healing in the elderly and diabetic. There are well validated diabetic mouse models that faithfully mimic human diabetic delayed wound healing. By contrast, there are no suitable in vitro or ex vivo models of diabetes (which is a whole-body disease). The ultimate goal of our research is to move our findings forward into clinical studies, testing newly identified pathways and drug targets for poor healing wounds. In vivo safety and efficacy data is currently a pre-requisite for obtaining ethical approval for first-in-man clinical studies.

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

Our group routinely use a range of in vitro and ex vivo models alongside in vivo studies to comprehensively evaluate the wound healing process. This is also essential to maximise the likelihood that effects shown in mice will also translate to the clinic. The assays that are available complement, but cannot currently fully replace, in vivo studies. They can however reduce the number of animals needed.

In Vitro Assays

We routinely use a wide range of in vitro wound healing assays when possible. These include scratch migration assays with different types of human skin cells, assays using human immune cells (e.g. to measure their ability to kill bacteria), assays to measure blood vessel formation, and assays that measure the growth of cells and their ability to make collagen and other skin proteins.

Co-Culture

To better model the complexity of actual wounds, it is possible to grow different cell types together. These models include growing two different cell types in close proximity so they can share secreted factors and communicate with each other (co-culture).

Skin Equivalents

Building upon the concept of co-culture, it is possible to grow very simple skin-like structures in the laboratory that contain some of the cell types found in skin. These are known as skin equivalents. Theycan be wounded and will undergo some of the processes observed during in vivo wound healing but they still lack many important components of actual skin and the wound healing response.

Ex Vivo Wounding

A relatively new model for skin and wound studies involves culturing actual human skin in the laboratory for up to seven days. This skin can be obtained from surplus material following surgical procedures. Ex vivo skin can be wounded and will heal to a certain extent outside the body. But again, it lacks several of the key processes involved in wound healing (such as a circulating immune cell response).

Pathological Wound Healing

Each of the above models can be modified to mimic poor healing, for example by using cells and tissues from aged and diabetic individuals, or by culturing cells in high glucose to mimic the diabetic environment. However, progress in this area is extremely limited and no models fully replicate ageing and diabetic wound healing.

Why were they not suitable?

We do use almost all the above non-animal models where we can. For example, we pre-screen drug compounds using in vitro and ex vivo models before moving to in vivo studies. However, it is widely acknowledged that none of the available models are currently able to fully replace in vivo studies.

There are broad limitations that apply to all the non-animal models. These include:

The inability to evaluate whole body effects on wound healing. Most models use individual cell types in isolation rather than evaluating how they interact together in whole tissue.

The inability to give drugs that affect the whole body (e.g. oral tablets).

The short nature of non-animal models. Human wound healing can take up to two years, while these models generally only last up to seven days.

These models also have limitations when considering ageing and diabetes. Ageing and diabetes change cells throughout the entire body. They also alter the way that cells interact with each other, and they change the environment that cells inhabit in ways that are too complex to mimic in currently available non-animal models.

Reduction

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

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

We estimate to use 1000 mice in our breeding protocol over the course of the five-year licence. This equates to a continual maintenance of four breeding pairs which will collectively produce 200 mice per year.

We estimate to use 1080 mice in our wounding protocol over the course of the five-year licence. An average wounding experiment will use 36 mice with 6 mice per group (range from 24-48 mice per experiment) and we envisage conducting one wounding experiment on average every two months. This equates to 216 mice per year.

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

Literature searches will be performed to determine the safety of proposed drug treatments for use in animal experiments, and their safe dosages. Safe drug treatments will first be screened (within a range defined from the literature) using our in vitro human wound assays and our established ex vivo human skin wounding model. This will allow us to screen a range of drugs at different concentrations and using different dosing strategies to find the most effective treatments and optimal dosing regimens for use during in vivo experiments. Using this pre-screening technique in non-animal models will significantly reduce the number of mice required for in vivo wounding experiments. The advantages of this approach are two-fold. Firstly, it demonstrates proof of effectiveness prior to undertaking in vivo studies, and secondly, it increases the likelihood that observed in vivo effects will translate into human clinical studies.

We have consulted the NC3R's Experimental Design Assistant in planning our studies, along with the PREPARE guidelines. In all experiments, standard protocols will be used. These include all relevant control groups, treatment randomisation with each mouse being an experimental unit, time-matching of control and experimental groups, and matching for gender, age and weight. We will not use sham surgery controls. Data from pilot studies will be included as experimental data where possible to reduce the number of mice used in subsequent studies. For example, if a pilot study using 3 mice per group showed that 8 mice per group were required to gain significant findings, the follow-on study would only use 5 mice per group and would be combined with the pilot study to give 8 mice per group.

All analysis will be carried out blinded so that researchers do not know what groups each sample was placed in. Interventions and outcome measures will be optimised to validate the study hypothesis with the minimum number of animals. We will photograph wounds throughout the experiment to assess healing over time and we will only collect the mice at specific time points post-injury. By photographing wounds and only collecting mice at one or two healing time-points, we will significantly reduce the number of mice that we will use (as we will not need to collect animals each day/every few days). We will collect mice at time-points post-injury that will be the most informative based on our extensive experience of the mouse wound healing response. We will also maximise the amount of tissue we collect from each animal, collecting samples for cell isolation and using two wounds per animal, with each wound collected and evaluated for multiple outcome measures. We follow a standard protocol for tissue collection and use established laboratory procedures.

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

Extensive literature searches have been carried out to avoid the unnecessary repetition of experiments. The work of others in the field will be constantly reviewed by the research team and disseminated to every member of the group. We will reduce experimental variation significantly by extensive training of investigators, the use of optimised techniques and by reducing factors such as age, genetic background and hair cycle stage.

We will use extensive in vitro and ex vivo pilot studies to optimise experimental treatment conditions prior to undertaking in vivo work. Where possible we will maximise the use of tissue obtained from experimental animals, sharing with other groups as appropriate. We

will continuously monitor breeding pairs and litters to ensure we do not overbreed mice. Mouse littermates that are not genetically altered will be used as experiment controls. Additionally, our institution routinely sends out a tissue sharing list to allow other researchers to make use of any surplus animal tissue.

Refinement

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

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

We will use mice exclusively as they are the least sentient species that are appropriate to address the aim of this project. We will use highly validated and optimised dorsal incisional and excisional acute wounds (small injuries to the skin on the back). We have chosen these wounding methods as they cause minimal discomfort compared to other models (e.g. burns, infection and pressure ulcers) but still allow us to address the aim of this work. There is an upper limit to the size of the wounds we will use to minimise the amount of inflammation and pain to the animal. In addition, mice will be put to sleep briefly for the procedure and will be given pain relief to reduce any post-surgery pain. From experience, pain experienced will be short-lived and mild and mice will make a rapid and full recovery, returning to normal behaviours within minutes (e.g. grooming and eating).

Mice will be singly housed after the procedure but only for the minimal time needed to ensure early wound healing is not affected by mouse interaction (maximum two weeks). Treatments will always be administered by the least invasive appropriate method, often topically applied to the wound.

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

We will use mice exclusively as these are the least sentient species that are appropriate to address the aim of this project. Other models are available ranging from embryos and developmental models (flies and frogs) through to large animals (pigs). A main focus of this project is understanding the factors that drive poor healing in ageing and diabetes. As such, embryonic and developmental models, while less sentient than mice, are not appropriate as they cannot model human ageing and diabetes. A potential less sentient model to use would be zebrafish. While zebrafish can be a useful model for laboratory research, there are a number of reasons why they are not a good model for human healing. Zebrafish skin structure is significantly different to human skin. The epidermis of the zebrafish is also covered with scales and lacks the hair follicles and glands present in the skin of mammals. In addition, wound healing stages in zebrafish occur in isolation, and do not overlap like in mice and humans. Another difference is that zebrafish heal with little scarring, while in humans, this only occurs under very specific circumstances (e.g. in the

mouth and during fetal development). As a result, the mouse has been selected as the least sentient species available to adequately assess aged and diabetic mammalian healing, but also has the advantage of being able to be easily genetically altered. Terminal anaesthesia is inappropriate for this study as we will be assessing wound healing over a time frame of days and weeks and cannot keep the animals under general anaesthetic for this amount of time as it is harmful and not practical.

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

After all procedures, animals will be monitored at least daily for signs of distress. Following surgery, animals will be placed in a clean cage inside a warm, ventilated cabinet and monitored until they recover. Analgesia will be administered as required to alleviate short-term discomfort to the animals. Extensive training will be provided to researchers undertaking experiments to ensure standardisation of the wounding technique and to ensure awareness of animal welfare and humane endpoints. Any treatments will be administered using the least invasive method to reduce harm to the animals.

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

We will follow the PREPARE guidelines to design and implement experiments in the most refined way. There is no official best practice guidance for wound studies per se, but there are a number of recently published methodological reviews (e.g. Rhea and Dunnwald, 2020, J Vis Exp) that we will use to establish standard operating procedures and ensure best practice. We will continuously review our protocols and procedures in line with updates to the PREPARE guidelines and published literature.

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

We regularly attend international conferences and are part of a large network of in vivo researchers. Specifically, we have carried out extensive literature searches to ensure this project avoids the unnecessary repetition of experiments and we will keep up-to-date with the scientific literature.

We will always adhere to the ARRIVE Guidelines 2.0 (du Sert et al., 2020, Plos Biology) when publishing and the PREPARE guidelines when designing and implementing experiments (Smith et al., 2017, Lab Animal). We subscribe to the NC3R's, FRAME and Animal Free Research UK newsletters and keep abreast of topical advances in 3Rs research and policy. To ensure that the research team are kept up-to-date with the latest developments in the 3Rs, we will hold regular group meetings, attend institutional seminars and review pertinent case studies.

32. Imaging of Cell Therapy In Tumour Models

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cancer, Therapy, Imaging, Stem cells, Immune cells

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to evaluate cell therapies for the treatment of cancer and compare them to other available cancer therapies.

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

Why is it important to undertake this work?

Cancer affects 1 in 3 people worldwide, with incidence rates projected to rise by 2% in the UK between 2014 and 2035. Cell therapies such as chimeric antigen receptor (CAR) T cell therapies have shown remarkable results in patients with blood borne cancers but have shown highly variable results in patients with solid tumours. This program of work will develop imaging biomarkers to assess cell uptake and infiltration in correlation with tumour therapeutic readouts to aid the development of cell therapies for the clinic and compare their treatment out comes with those of available drug treatments (clinical gold standard) for known cancer types.

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

This project aims to develop new cell labelling imaging approaches to determine the effectiveness of cell therapies to cancer. We aim to produce cell labelling products capable of monitoring therapeutic cells throughout the body that can be translated to clinical practice. These, cell labelling products will provided new information on the fate of cells after injection which will benefit scientists and clinicians working on the development of cell therapies. We have already produced one cell labelling product that has been approved for a first-in-man study where it will be used to image a cancer cell therapy in humans with lung cancer. Due to the increase in the development of cell therapies for cancer I therefore expect the demand for these cell labelling imaging strategies to continue to grow over the next 5 years of this PPL in accordance with clinical need.

Expected outputs derived from this PPL:

Products:

New cell labelling imaging agents. New information:

Although cell therapies have been applied to hundreds of clinical trials very little is actually understood about where the cells go and how long they live for within the body. We aim to answer these important questions.

Publications and public engagement:

The data obtained from this work will be communicated by publications to scientists and by public science engagement events.

Clinical Translation:

The best cell labelling imaging products will be translated to the clinic for imaging cell therapy in human cancer patients.

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

The overall aim is to develop and test the safety of cell therapies in a range of tumour models to enable their clinical translation.

Who will benefit:

Basic science - The novel cell labelling products developed in this proposal will have a direct benefit for academic research scientists (both nationally and internationally) that are working on cellular therapies, medical imaging or the underlying scientific disciplines used to make the cell labelling products (e.g. chemistry, genetics).

Clinical translation – The ability to accurately track cells to their target site and monitor cell integrity over time provides crucial information on how effective the cell therapy will be and how long the cell therapy will last, which aids clinicians plan effective patient treatment plans.

Patient benefits: CAR-T cell and stem cell therapies are aimed at cancer patients with advanced cancer where all other therapies have failed. CAR-T cell therapy has already shown benefits in patients with blood cancers but we aim to apply this therapy to all cancer types. Whereas stem cell therapies have been shown to be effective in lung cancer which is one of the most lethal cancers in the world today.

Clinical investment: The global commercial cell therapy industry was estimated to have an annual turnover of £8.7 bn ($11.2 bn) in 2017 and is estimated to grow to £26.6 bn ($34,4 bn) by 2025. The UK regenerative medicine platform is also investing £45M over 8 years and is working in conjunction with the Technology Strategy Board's Catapult which represents £1bn of private and public sector investment across seven centres of which cell therapies is one. The charitable investment in regenerative medicine was approximately 20% of public funding between 2005 and 2009, and it is anticipated that charitable funding will also increase significantly.

Expected short and long term benefits.

Short term: To design and test new cell labelling products sensitive enough to be able to image cells throughout the whole-body and use them to assess the distribution of cells after transplantation and optimise their uptake in target organs by changing the injection route. We will identify the effectiveness of cell therapies in a range of tumour models and compare these outcomes to standard drug therapies such as chemotherapy. We will also identify potential safety considerations that could lead to long term health concerns.

Long term: To provide knowledge and clinical cell imaging products to aid the translation of cell therapies to clinic practice for the treatment of cancer patients.

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

This project is a continuation of a previous 5 year project which has been highly successful in the production of cell labelling products and publications. The work has facilitated numerous new collaborations with experts within the cell therapy and imaging communities as well as clinical translation. This project proposal will maintain these collaborations to ensure continuity between the old project and the new. New collaborations will be including the use of computational modelling, where data sets derived from our cell imaging can be used to train the computer simulation to predict cell behaviour thereby reducing the use of animals in our future research.

We will distribute our data through the most appropriate channels, such as by presentations at national and international meetings, publications and public engagement events.

Species and numbers of animals expected to be used

- Mice: 7000
- Rats: 500

Predicted harms

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

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

The mouse is the most appropriate model species for this investigation as they are the lowest animals in the evolutionary tree in which suitable models of cancer can be carried out. The project also requires the use of genetically modified mice strains where the mice have a compromised immune system to prevent the rejection of human cell lines. It is important to test our cell labelling products in human cells so that they are safe for use in clinical practice.

The rat will also be used in the case of protocols 1 and 3. These maybe wild type or athymic nude rats. Rats have previously been used as the main species of choice for brain metastases as their brain is bigger than the mouse with better image resolution. They also have a bigger blood pool which allows for serial blood sampling for quantification of tracer pharmacokinetics which is essential for translation into the clinic. This quantification in rats is more accurate mice. As protocol 1 covers the development of tracers in control animals including pharmacokinetics, and 3 covers the brain metastases model, rats will only be used in these two protocols.

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

In general animals will receive an injection of tumour cells to induce tumour formation, which may be via a surgical procedure. All surgical procedures are short procedures (up to 20 minutes) and are used by trained experts. The use of surgical procedures has also been refined by the use of ultrasound guided techniques where possible to aid animal recovery.

Animals are scanned using non-invasive imaging (1 to 2 times weekly up to 1 hour) to monitor tumour development. Once tumours have reached treatment size (typically within 2- 3 weeks) the animals will be randomised into groups for treatment.

Tumour bearing animals will be given either single or multiple doses of therapy (cell or drug) dependent on the route of injection e.g. intravenous may be given multiple times (dependent on the dosing table) whereas injections via a surgical route will be given only once.

Animals will be continued to be monitored non-invasively via imaging (see above) to follow treatment outcomes such as changes in tumour size and tumour cell death. The typical duration of experiments will be 1 to 2 months depending on the growth rate of the tumour.

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

Animals will experience tumour development and tumour treatment and may experience weight loss. Animals will be monitored 2 to 3 times a week and any animals experiencing 10% weight loss will be weighed daily. As tumour growth can also affect animals weight, they will also be assessed by their body condition and behaviour.

Surgical procedures and cell injections can cause internal bleeding or vascular occlusion. Animals are assessed rigorously during procedures and directly after upon recovery, any animal showing evidence of bleeding or vascular occlusion will be humanely culled immediately.

If any animal reaches 15% weight loss with at least one other clinical sign, body condition score <2/5 or exhibits any evidence of with 24 hours: neurological changes such as seizure/fitting, respiratory distress, marked piloerection with hunched posture, reduced mobility, pallor, persistent ocular/nasal discharge, or diarrhoea, the animal will be humanely killed.

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

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

Non-invasive imaging will be used to monitor tumour development and tumour response we have therefore refined our expected impacts/adverse effects as much as possible as we are able to identify small morphological changes in vivo prior to the onset of clinical signs, thereby using much milder disease.

All tumour models and injection routes are routinely used within the group and undergo constant refinement to keep the severity level and potential adverse effects as low as possible. The severity is maintained as moderate due to the need for tumour modelling, surgical implantation and non-invasive imaging.

Expected mortality: In previous experiments we observed a low (0-5%) mortality as a result of these experimental procedures (NB. mortality is mainly from cell injections rather than the experiments as a whole).

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

- Killed

Replacement

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

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

The aim of this project is to investigate the potential of different cell therapies to target and kill cancer throughout the body. Cell therapy is dependent on cell delivery and cell infiltration into tumour tissue which is highly affected by the solid tumour environment and which organ the tumour is situated in within the body. Cell assays cannot replicate all of the complexities of the solid tumour environment within a host making cell therapy unpredictable. This proposal aims to address this by applying imaging to monitor cell therapy in mouse models of solid tumours.

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

We use in vitro assays to enable us to identify the therapeutic potential of all cell therapies prior to moving into a mouse tumour model system. We also aim to use our imaging data to train computer generated model systems that can be used to predict cell therapy outcomes.

Why were they not suitable?

Although in vitro assays can replicate some of the events present in cancer they do not replicate the biological, chemical and mechanical properties of the body, organ or solid tumour tissue. To optimise cell delivery and infiltration to solid tumours their behaviour needs to be monitored within a host with underlying disease. For this reason, there is no in vitro alternative to animals that we can consider.

Reduction

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

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

We estimate the numbers of animals we will use from data obtained from previous experiments or from the literature. However, when working with novel tracers, tumour cell lines and cell therapies, means and standard deviations will be taken from preliminary pilot studies consisting of 3 to 5 animals. The experimental design of these preliminary pilot studies will include appropriate controls to allow the detection of the effect of change of individual variables. Appropriate statistical analysis methods are then applied to the data obtained to obtain our estimates.

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

All in vivo studies are designed with assistance from the NC3Rs Experimental Design Assistant.

Monitoring animals by in vivo imaging makes a major contribution to the reduction in animal numbers as each animal can be scanned over multiple time points following disease progression and therapy. This allows us to use fewer animals to achieve the same statistical power as conventional designs in which cohorts of animals are required for each time point. Therefore, a classic experiment where 5 animals are humanely killed at 3 time points for histological analysis would result in 15 animals per group. Whereas, with imaging this can be reduced to 5 animals per group as each animal is scanned to obtain the results at the 3 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?

In establishing a new cell labelling product, tumour model or assessing a new cell therapy a small number of mice (3-5) will be used as a pilot study where the mice will be assessed and adjustments made to make sure that models are robust with minimum severity before being applied to a full experimental cohort. This will reduce the total animal number and constantly refines our protocols.

We aim to use the imaging data derived from this project for computer modelling where our 3 dimensional data of cell distribution will be used to train the computer simulations which will be used to model and predict cell behaviour.

Tissue will be taken for histology, immunohistochemistry and ex vivo validation to ensure our therapeutic cells are within the tumour tissue and to confirm the therapeutic effect shown by imaging. We ensure that any tissues generated from experiments are archived and stored appropriately therefore ensuring that unnecessary repetition of experiments is not necessary.

As part of good laboratory practice, all experimental designs including data analysis will be approved by the PPL holder before the experiment can start, and a protocol record for each experimental animal has to be kept by the PIL holder which will include the description of the experimental steps, treatments, and animal monitoring.

Refinement

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

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

The mouse is the most appropriate model species for this investigation as they are the lowest animals in the evolutionary tree in which suitable models of human cancer can be carried out, no use of species phylogenetically closer to humans is proposed.

The tumour models we have chosen are well documented and well validated to produce reliable results within a moderate severity band. The tumour inductions are short procedures, and therefore the animal recovery is rapid and no adverse effects are expected. Visible tumours will be measured by callipers.

Whereas internal tumours will be monitored and sized by rapid MRI, CT, BLI or ultrasound scans to generate growth curves. Due to this, tumour growth in all settings, will be closely monitored and will not be allowed to reach a point where it may cause any discomfort to the animal.

The rat will also be used in the case of developing of cell tracking techniques within the brain. These maybe wild type or athymic nude rats. Rats have previously been used as the main species of choice for brain metastases as their brain is bigger than the mouse with better image resolution. They also have a bigger blood pool which allows for serial blood sampling for quantification of tracer pharmacokinetics which is essential for translation into the clinic.

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

As stated above the mouse is the most appropriate model species for this investigation as they are the lowest animals in the evolutionary tree in which suitable models of human cancer can be carried out.

Host microenvironmental changes during animal development will influence tumour growth and cell behaviour which will continuously alter until adult hood adding unknown variables to the data and may result in irreproducibile data being generated. Adult animals have reached full organ development and therefore removes this variable, also due to size of the animal proposed adult animals are necessary for the procedures required for tumour implantation, cell injection and imaging to be conducted using refined and practised methods.

Tumour models will be generated in adult mice at ages 6 to 8 weeks to ensure tumour reproducibility and cell therapy as our data has indicated that increased age effects tumour implantation, animal recovery and immune status in some animal strains.

The aim of this project is to assess cell therapy in tumour models which are conducted over a period of weeks not hours we are unable to use terminally anaesthetised animals to conduct all aspects of these experiments.

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

Animals undergoing surgical procedures will receive peri-operative analgesia. Animals will be monitored intensely after surgical procedures and after cell implantation to check for clinical signs. Directly after the procedure the animals will be constantly monitored until they have completely recovered from the anaesthesia. Animals will then be monitored every 20 minutes for the first hour, then every 1-2 hours within working hours and first thing the next morning. If no clinical signs are shown and the wound shows no infection or bleeding then the animals will be monitored 2 to 3 times a week.

The procedures within this project are run routinely and, in our experience, animals recover rapidly after surgery and very rarely require further post-operative analgesia after 24 hours except in the case of suture failure where the wound has needed to be re-sutured (within first 24 hours).

When assessing animals after surgery and tumour implantation the assessment of pain will be assessed using the "grimace scale" and will form part of an animals clinical signs assessment. A post-surgical/cell implantation monitoring sheet will be completed each time the animal is checked over the first 24 hours using the animal's unique identifier code.

The monitoring sheets are specific to each of the protocols and cover the expected clinical signs for that protocol.

Animals will be weighed 2 to 3 times a week. Any animal that reaches 10% weight loss will be weighed daily to make sure that it does not exceed the 15% weight loss.

Animals will also receive ultrasound guided injections where possible rather than surgical routes. This reduces any risk to infection and vastly increases animal recovery and limits pain.

To maintain that animals are not fasted for longer than designated time frame and if food cannot be removed at the desire time, we will make sure that a measured number of pellets will be left in the hopper.

Lastly, we will constantly refine our protocols during the 5 year duration being guided by our imaging developments and the support of the NACWO and NVS.

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

All in vivo studies are designed with assistance from the NC3Rs Experimental Design Assistant and will abide by the ARRIVE guidelines. Myself and my group regularly consult the NC3Rs resources available in conjunction with our establishments guidance, training and meetings.

However, as mentioned previously imaging measurements can often be used to refine protocol with milder disease than with other assessments by providing earlier humane endpoints and rigorous inclusion/exclusion criteria. By working closely with the NACWO and NVS we aim to incorporate imaging mediated refinement where possible.

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

All PPL holders must attend yearly 3Rs meetings. We are committed to the principles of the 3Rs and will continue to seek the support of local NC3Rs representatives.

With the support of our Named Animal Care and Welfare Officers (NACWOs) and Named Veterinary Surgeons (NVSs) I strived to use our imaging methods to refine our tumour models and surgical protocols i.e., develop ultrasound guided injections rather than surgical implantation.

When using imaging measurements of small morphological changes in animals, it is often possible to use milder disease than with other assessments. Non-invasive imaging of animal disease models can therefore provide earlier humane endpoints and rigorous inclusion/exclusion criteria.

33. Investigating how genomic imprinting influences brain and behaviour

Project duration

5 years 0 months

Project purpose

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

Key words

Behaviour, Brain, Imprinted genes, Pregnancy, Neurodevelopment disorders

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to investigate how genes subject to genomic imprinting influence brain and behaviour, and how expression of these genes may in turn be influenced by the maternal environment before and just after 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?

In animals, genes that are subject to genomic imprinting (referred to as 'imprinted genes') are unique to mammals. Although they represent a small proportion of the genome - ~200

genes of a total ~22,000 - correct activity, or expression, of imprinted genes is absolutely critical for normal development to proceed. Unsurprisingly, mutations that affect imprinted gene expression in humans have been linked to several genetic disorders, including many neurodevelopmental disorders such as Prader-Willi and Angelman syndromes.

We want to understand how imprinted genes influence brain and behaviour. Many imprinted genes are expressed in the brains of mammals, and can influence behaviour directly. However, our previous work has shown that imprinted genes expressed in other tissues, such as the placenta, can also influence behaviour via indirect mechanisms. This project will investigate both the direct and indirect action of imprinted genes on brain and behaviour. In addition, we will also examine how the pre- and post-natal maternal environment may alter the expression of imprinted genes, which in turn has knock-on effects on brain and behaviour.

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

The main outputs will be extensive datasets, many of which will be included in scientific research publications.

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

The main beneficiaries of this work will be the research community, particularly those involved in understanding imprinted genes. Additionally, as much of the work outlined here has a direct link to neurodevelopmental disorders (e.g. Prader-Willi syndrome) it is hoped that this research will have translatable benefits (such as the guidance of best practice and/or development of therapeutics); this will be helped by our relationship with the patient charity, the Foundation for Prader-Willi Research (US).

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

We will strive to make all our data freely available via the use of project planning and data repositories such as the open science framework (osf.io). Preregistering projects will allow scrutiny of methodology and statistical methods before the experiments have begun, thus further refining any experiments involving animals. Similarly, where possible we will make use of registered reports, which will lead to further refinement of methodology before the experiments start, and will also mean that any data generated, whether positive or negative, will be published. All data generated from these projects will be made freely and openly available.

We will also adopt open publishing practices where possible. In addition to using preprint servers such as bioRxiv, we will, funds permitting, publish our work as Gold open access articles.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

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

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

It is necessary to perform whole animal studies to achieve the experimental aims, since in all instances integrated physiological systems are affected. Behaviour is an emergent property of brain function, involving co-ordinated activity both within and external to the CNS. It is also not possible to model pregnancy using cell lines or any other in vitro system. Pregnancy and placental development are features of mammals generally not observed in other vertebrates. Consequently, it is not feasible to use a non-mammalian model.

Mice are the best experimental model for this project since their physiology is well studied, they are genetically modifiable and they are the lowest vertebrate group appropriate for studying fetal growth and pregnancy.

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

The aim of this project is to understand the brain and behavioural role of a unique group of mammalian genes called the imprinted genes. Typically, we will modify the expression of one or more of these imprinted genes and look at the consequences of this manipulation on brain development and function. To do this we subject an animal to a number of tests that examine basic behaviours such as activity levels or anxiety. An animal may then also be tested on a more complex test of cognition, examining memory, attention or impulse control. In total, all these experiments may last 6-8months. Another particular focus of this project is parenting behaviour. Here we would monitor parenting behaviour remotely via video cameras in the mouse's home cage or examine parenting behaviour of males and females using a series of tests that measure the time taken to perform basic parenting behaviours such as nest building or gathering the pups into the nest. These experiments last 3-4 weeks in total. There are possible links these groups of tests. For instance, a pup exposed to poor parenting behaviour may then be tested on aspects of cognition when adult.

In addition to genetic alteration, we know that the expression of imprinted genes is also affected by the environment in early life. As part of this project, we will also examine the effects of certain adverse environments on imprinted gene expression and subsequent behaviour. This could be an altered maternal diet during pregnancy (duration of 18.5 days), or being exposed to poor parenting behaviour whilst a new born.

We will also explore the possible links between the genetic alterations and early life environment. For instance, a pup exposed to poor parenting behaviour may then be tested on aspects of cognition when adult.

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

The majority of impact seen in the animals used on this project will be behavioural in nature, and many may not display any abnormal behaviour until probed to do so; i.e. normal functioning within the homecage will be unaffected, and behavioural abnormalities will only emerge upon specific testing.

These abnormal behaviours are likely to be present throughout the lifespan 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)?

The majority (~90%) of the genetically altered mouse lines we will use are classified as “mild”, with a minority classified as “moderate” due to the survivability issues. In addition, we aim to examine the consequences of altering the maternal environment, in particular maternal diet, on gene expression in the offspring brain. We have refined this protocol to minimise harms to the pups when born, but generally this is classed as moderate.

Most of our behavioural testing is either unregulated or classed as mild (~50%). However, to motivate animals to learn and perform some of test of cognition we will be restricting their normal access to food or water. This has no long lasting effects, and indeed we have shown it can prolong the lifespan and health of the animal, but nevertheless it is as moderate (~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?

It is necessary to perform whole animal studies to achieve the experimental aims, since in all instances integrated physiological systems are affected. Behaviour is an emergent property of brain function, involving co-ordinated activity both within and external to the central nervous system. Consequently, changes in gene expression processes may affect brain functioning, and hence behaviour, at multiple levels. In particular, we are investigating parental behaviour, and the link between gene expression and maternal diet on changes in offspring behaviour. It is not possible to model pregnancy using cell lines or any other in vitro system. Finally, genomic imprinting is a uniquely mammalian phenomena in animals, therefore it is not possible to use other model species.

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

We considered using in vitro, or cell culture models. Where possible, we will use these cell culture to directly examine the effect of manipulating a gene on neuron physiology.

Why were they not suitable?

Much of this project is focused on the physiological interactions between mothers, placentae and foetuses; and then, following birth, the relationship between mum and/or dad, and the newborn offspring. Currently, this aspect of behaviour, which involves complex co-ordinated interaction both within, and external (i.e. from the newborn pup) to the central nervous system, cannot be modelled 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?

The number of animals we estimate will be used in the course of this project is based on our experience and knowledge of breeding the necessary GA lines, and the numbers of animals required for a given experiment.

This number deviates from the simple sum of the animals used in each protocol (2000+200+1000+2000) because some animals will be used in more than one protocol. For instance, some animals initially bred under Protocol 1 will then have their parenting behaviour tested under the aegis of Protocol 3. Similarly, animals that were exposed to abnormal maternal diet in utero in Protocol 3, may then go on to be tested for behavioural abnormalities as adults under the aegis of Protocol 4.

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

As a routine measure, we carry out power calculations based on previous experimental data, generated by ourselves and others, in order to optimise the size of the experiment. To aid this, and our experimental design generally, we use the Experimental Design Assistant provided by the NC3Rs.

We also have the benefit of experience in making initial estimates of the numbers needed in a behavioural study to obtain statistically significant differences. We examine the reliability of our conclusions using a range of statistical tests, suitable for both parametric and non-parametrically distributed data. We make routine use of multifactorial methods (e.g. ANOVA, ANCOVA) that combine the power to detect significant differences with the minimum number of subjects. We have substantial experience in the use of statistics and we take advice from, and interact closely with, University statisticians, as well as making use of external sources of guidance (e.g. 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?

We have a number of close collaborators. Because our research addresses similar questions, but looking at different biological processes, where possible we reduce our numbers by combining studies.

Example A: Breeding and maintenance of GA lines used by both our group and collaborators (e.g. Cdkn1c, Syn-cre), is carried out only by one group.

We cryopreserve lines when not in use.

We maintain all our breeding lines in a single facility so that lines are not duplicated

Where appropriate, we use animals that are not GA but have been born as part of the process of generating GA animals that are not needed, as controls. These animals may be used in new mattings (Protocol 1) reducing the requirement for wild type animals from wild type mattings.

Where appropriate, we will use non-invasive techniques to examine brain function (eg MRI scanning). MRI also allows longitudinal studies using the same animals, thus reducing 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.

Mice are chosen as an experimental model since their physiology is well studied, they are genetically modifiable, and they are the lowest vertebrate group appropriate for studying fatal growth and pregnancy.

Many of the behavioural methods used in this project are measures of spontaneous natural behaviours in response to a stimulus. Of those where training and learning is required, we only use appetitively reward tasks.

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

Genomic imprinting is a uniquely mammalian phenomena in animals, therefore it is not possible to use other model species such as fruit fly or fish.

In addition, a large part of this project is focused on the physiological interactions between mothers, placentae and foetuses; and then, following birth, the relationship between mum

and/or dad, and the newborn offspring. Currently, this aspect of behaviour, which involves complex co-ordinated interaction both within, and external (i.e. from the newborn pup) to the central nervous system, cannot be modelled in a cell culture system.

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

We routinely genotype animals from hair samples. Where possible, we will avoid ear snips for genotyping and use hair sampling. For example, experimental cohorts (behavioural and neural studies) are genotyped from hair samples. Whilst genotyping from hair is appropriate for experimental cohorts that will be re-genotyped at the end of testing, a robustly reliable genotyping method is required for animals used for breeding. Consequently, these animals will continue to be genotyped from ear-snip samples.

Together with collaborators we have refined the manipulation of maternal diet whilst pregnant by returning them to a normal basal diet at E18.5 (just prior to birth). For the low protein diet in particular, this modification of the procedure reduces the risk of cannibalism of the pups by the mother shortly after birth but still produces the changes in offspring brain gene expression and behaviour we aim to study.

Environmental enrichment is provided for all animals, which can include cardboard houses or cardboard tubes, chew sticks and the avoidance of singly housed animals where possible.

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

NC3Rs publications and blogpost; best practice guidance from AWERB and NACWOs.

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

In addition to local advice from the AWERB and NACWOs, we will also regularly engage with NC3Rs via the Regional Programme Managers.

34. Neuronal communication in fish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

zebrafish, vision, neuron, synapse, olfaction

Animal types	Life stages
Zebra fish	embryo, 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?

Our aim is to understand how neural circuits in the retina and other brain centres process sensory information.

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

Why is it important to undertake this work?

This work is expected to improve our understanding of nervous system function in three main areas. First, the basic processes by which neurons communicate at the synapse. Second, the way these processes transfer visual information in the nervous system. Third, the manner in which visual, olfactory and mechanical stimuli interact in the brain ("sensory integration"). We also expect that the methods we develop will be applicable to other areas of the brain, for instance allowing us to investigate the processing of olfactory signals, or motor signals in the cerebellum. Previously, it was only possible to monitor the activity of a few synapses at any one time, but we have made a major advance by monitoring the activity of hundreds of different synapses and neurons simultaneously. This will allow us to investigate how a large number of synapses cooperate to carry out an information-processing task. This work is immensely worthwhile because it directly addresses a key

function of the nervous system: the transformation of signals as they are transmitted between neurons.

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

This work is expected to improve our understanding of nervous system function in three main areas. First, the basic processes by which neurons communicate at the synapse. Second, the way these processes transfer visual information in the retina. Third, the manner in which visual, olfactory and mechanical stimuli interact in the brain ("sensory integration"). We also expect that the methods we develop will be applicable to other areas of the brain, for instance allowing us to investigate the processing of olfactory signals, or motor signals in the cerebellum. Previously, it was only possible to monitor the activity of a few synapses at any one time, but we have made a major advance by monitoring the activity of hundreds of different synapses and neurons simultaneously. This will allow us to investigate how a large number of synapses cooperate to carry out an information-processing task.

This work is immensely worthwhile because it directly addresses a key function of the nervous system: the transformation of signals as they are transmitted between neurons.

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

These outputs will benefit many people by adding to our understanding of sensory processing in the brain. This knowledge may be useful in understanding causes of blindness and other diseases of the nervous system as wall as providing examples for bio-inspired robotics.

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

We will maximize the outputs by collaborating directly with several other laboratories in Europe and the US; by communicating our work through seminars and meetings; by publishing papers in scientific journals and through various websites, including our own.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): A maximum of 42000 over 5 years.

Predicted harms

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

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

Larval zebrafish are an excellent animal for studying the senses of vision and smell, for two major reasons. First, we can manipulate genes in this animal in a relatively straightforward way. Second, the larva is relatively transparent so that we can use specialized microscopes to observe the activity of neurons and synapses as the animal responds to stimuli. Zebrafish are an excellent model system for vision in humans because there is a great deal of similarity in the two visual systems at the anatomical, molecular

and physiological levels. Fish are therefore also a model system that is relevant to understanding medical conditions that interfere with vision in humans.

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

Zebrafish will be bred and we will collect the fertilized eggs, into which we will inject DNA to make a transgenic animal. This DNA normally causes the offspring to express a fluorescent protein which is harmless to the animal but which generates optical signals when neurons and their synaptic connections are activated. When these genetically modified animals them selves breed, we use their larvae 6-9 days after fertilization. The larvae are anaesthetized and then placed in a specilaized microscope that allows us to observe changes in the fluorescences of proteins in neurons. This experiment will typically last 1-2 hours, at the end of which the larval zebrafish is killed humanely.

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

- No common adverse effects are anticipated.

Expected severity categories and the 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 have been categorized as "mild" for all animals.

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

- Killed

Replacement

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

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

There is no substitute for the retina or brain to understand how the retina or brain works in processing visual information. The fish visual system represents an excellent alternative to mammals for the study of many aspects of nervous system function that are conserved among vertebrates.

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

It might be possible to study small parts of the general question (such as the basic mechanisms by which synapses work) using neurons in culture. But this is not the major question that this project sets out to answer of the project.

Why were they not suitable?

Cultures of neurons cannot see or smell and are not connected in the way the visual and olfactory systems are in the intact 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?

Procedure 2 will involve a maximum of 12000 fish, calculated as 8 scientists using an average of 7.5 fish per week for 40 weeks per year over 5 years.

Procedure 1 will involve a maximum of 30000 fish, based on 40% (12000) proceeding to Procedure 2. About 60% of larvae grown beyond 5 days post-fertilization (when they come under the Act) do not proceed to Procedure 2 because of the limited number of experiments that a single scientist can do per week and the fact that we cannot control how many eggs are layed and fertilized and cannot use fish beyond 9 days post-fertilization.

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

During the design phase we have carefully chosen the lines of zebrafish so that they can be used by the largest number of scientists working on this project (see below). 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 (e.g. when injecting a drug into the eye).

Cryopreservation will be used to preserve important lines and remove the necessity to hold stock for extended periods.

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

Scientists will breed a particular line of fish to be shared with others. A "good" breeding in Procedure 2 might produce 40 larvae of a particular type on a particular week. Two scientists will share these fish, so that an average of 15 are used per breeding rather than an average of 7.5.

Refinement

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

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

Larval zebrafish will be used, which are generally considered less sentient than juveniles or adults because their nervous system is still developing. They will be bred from adults kept in well-maintained aquaria and when used for experiments they will usually be under terminal anaesthesia. For embryos at motile stages of development before they are protected by the Act, we still use anaesthetic for all operations and embryonic surgery. MS222 (also known as tricaine) is currently the anaesthetic of choice but we will also investigate the utility of etomidate, which has been reported to produce less obvious aversive effects. The anaesthetic regimen is similar for fish used after they are protected by the Act: the anaesthetic is added (with antibiotics) to the sterile water bathing the fish at appropriate concentrations (2 parts per thousand for MS222 applied to fish at 5-9 dpf). The animals quickly become insensible, and so analgesics are not used.

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

We have to use a vertebrate species because the structural plan of the visual system is very different in invertebrates.

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

For embryos at motile stages of development before they are protected by the Act, we still use anaesthetic for all operations and embryonic surgery. MS222 (also known as tricaine) is currently the anaesthetic of choice but we will also investigate the utility of etomidate, which has been reported to produce less obvious aversive effects.

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

We will follow "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).

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

We will stay informed by:

Using the National Centre for replacement, refinement and reduction (NC3Rs) as source of help, especially regular e-mail bulletins (NC3Rs News) that are forwarded to us by our NACWO.

Good communication with staff in the Biomedical Research Facility (BRF) who attend industry conferences and are in regular contact with staff in other facilities in the UK, as well as our Home Office Inspector and Named Veterinary Surgeon.

Meetings of BRF staff and users.

Implementation of advances will occur in consultation with our NACWO, Home Office Inspector and Named Veterinary Surgeon. All members of the lab working under this license will be involved.

35. The neurobiological bases of mental health across the life span

Project duration

5 years 0 months

Project purpose

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

Key words

Neuroscience, Mental Health, Neurodegeneration, Alzheimer's disease, Behaviour

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

To study the neurobiological mechanisms underlying both normal brain function and disorders, and how risk/resilience factors interplay to drive changes in neural processing and behaviour.

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

Why is it important to undertake this work?

The World Health Organisation has estimated that a quarter of the world's population will suffer from mental or neurological disorders in their lifetime. There are key points in the lifespan when people are especially susceptible, for example in adolescence (mental health conditions account for 16% of the global burden of disease and injury in 10-19 year

olds) and our ageing population (the risk of dementia increases with every decade). There are many factors, both environmental and genetic that can increase risk or resilience to mental health disorders. Studying the neurobiological mechanisms underlying both normal brain function and disorders, and how risk/resilience factors interplay to drive changes in neural processing and behaviour, will provide insight into the burdens placed on the human brain and strategies both pharmacological or behavioural that could be used to mitigate damage and build resilience to mental health disorders and disease.

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

This project will output academic publications which will help other researchers to understand the mechanisms of disease to eventually lead to the new therapeutic strategies. As we develop new behavioural protocols and tests we will publish these, both in academic journals and as an open access resource to give other researcher free access to new techniques. Through establishing how genetic and environmental factors impact mouse behaviours (associated with mental health disorders) , and understanding the neurobiological mechansims driving these differences we will help to develop understanding of mental health disorders across the lifespan, which can be presented to the public and other research groups in publication or presentation formats.

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

Academic publications: Publications are crucial to allow sharing of our techniques with other researchers. This will improve reproducibility of science between laboratories and help grow the evidence base.

There are multiple levels of benefits to this. For example, with the proposed work on APOE and Alzheimer’s disease:

Short term (2-3 years): We aim to establish a time course for how APOE increases AD risk to find targetable timepoints for treatment. We also aim to establish some of the mechanisms involved in this risk at different levels of study (i.e. from whole brain to individual cells)

Medium term (3-5 years): We aim to identify and test new methods to alter APOE genotype or alter APOE-associated changes at different levels of study (i.e. how to target APOE in the whole brain vs effects on individual cells).

Long term (10+ years): Alzheimer’s Disease and other dementias are becoming an increasing health burden as life expectancy has increased. Recent statistics suggest that a third of people born in 2015 will suffer from dementia. A treatment that might prevent the onset of dementia by 5 years would reduce this number by a third. Up to 30% of the population carry an APOE4 allele and thus their risk of AD is doubled. If we could reduce/eliminate this risk it could have a large effect both on individual suffering and the economic burden of AD. This project seeks to establish the mechanism by which APOE4 confers this risk and develop and test strategies / treatments to prevent or reverse it, with the long-term potential of a new effective therapeutic strategy for Alzheimer’s Disease.

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

- Using multiple avenues of dissemination so it reaches a wide range of audiences. As well as academic publications, present the data in different forums, to demonstrate the significance to all stakeholders, from academic researchers to drug discovery and clinical researchers, policy makers and the educated public.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

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

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

Mice are going to be used in this project because of their comparability to humans as a model for behaviour and brain structure/function. The biological markers of ageing have been, to some extent, mapped between mice and humans. This, in addition to the fact that mice have a much shorter lifespan than humans and can therefore be studied more quickly, provides good motivation for using mice in biological research. Finally, we are taking a lifelong approach to try to get the most information from individual animals as possible, minimising the number of animals and giving us insight into the lifespan ageing process.

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

The majority of animals used in this project will undergo repeated mild stressors such as handling, participation in behavioural procedures (e.g. tests of memory and attention) for periods of several months. Some animals will also be administered with substances which may cause mild discomfort and experience stress related to the substance.

Other animals may undergo a combination of the above-mentioned procedures in addition to surgical procedures. All surgical procedures are carried out under anaesthesia with best practice including post- operative care and pain management. Any suffering exceeding that which is set out in the protocol for these procedures will be monitored and necessary interventions (such as treatment, or in severe cases, culling) will be performed.

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

Protocol 1: we do not expect any adverse harms to the animals in protocol 1, as this simply involves maintenance and breeding as standard procedure.

Protocol 2; Animals in protocol 2 may experience mild weight loss and acute stress due to the procedures carried out. However, the distress caused is expected to be mild and transient.

Protocol 3; Animals in protocol 3 will be exposed to mild handling and testing stress due to repeated human contact and placement in testing apparatus. The cumulative effect of

this is expected to be mild as, for the most part, animals are exposed to appetitive cognitive tasks which may be considered environmentally enriching. Animals may experience mild weight loss due to food restriction. Animals may also experience cumulative moderate stress due to repeated administration of drug substances. However, animals will be regularly monitored for signs of distress and will be culled if exceeding the set severity limit for protocol 3.

Protocol 4; Animals may experience the stressors and harm outlined in protocol 3, in addition to exposure to invasive surgery. While it is unlikely, animals may experience post-surgical complications such as infection, pain, or adverse behaviour. These events will be monitored for and animals will be treated as appropriate (e.g. provided post-operative pain relief). The expected severity outcome for animals in protocol 4 is moderate, and animals will be monitored for signs of exceeding this severity limit with repeat testing.

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

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

Subthreshold - 30%

Mild - 30%

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?

There is no non-animal alternative for behavioural studies to study cognition and behaviour. Study of genetic changes in humans is difficult due to high variability between individuals, and difficult to control lifestyle factors. Using mice allows us to control for these issues and test hypotheses across the lifespan. In addition, use of mice provides us with invaluable tissue (e.g. brain) at different times across the lifespan, which would otherwise be inaccessible. With access to this tissue, it is possible to test the mechanisms of disease that would otherwise be impossible in humans. Finally, mice are a good model system for human genetics and, to some extent, disease processes. Mice share a higher similarity in genetic structure and brain circuits to humans than lower organisms such as zebrafish or other model animals.

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

For behavioural studies there are no non-animal alternatives. For development of new genetic tools, we will begin in vitro and only transition to in vivo when we are confident that we are able to effectively manipulate or express the genes in question.

Why were they not suitable?

In vitro methods cannot substitute for live animal research where we analyse behavioural outcomes and the interaction of neurobiological mechanisms at a 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?

These numbers have been estimated from both previous experience and future planning for breeding mice with relatively complex genotypes and a calculation of numbers of experiments and numbers of mice within experiments.

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

We perform statistical tests to assess how many animals we need to achieve reliable results. This is also based on prior experience in our and other laboratories as well as the scientific literature. We use repeated comparisons within individual mice where possible, as this can reduce the total number of mice needed and help us understand the within-individual effects we are researching. In our establishment we have access to statistical consultants to ensure decisions made about experiment size are appropriate.

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 strategies to breed specifically for the numbers of animals needed for given experiments. This allows us to reduce our numbers to a minimum. In addition, we perform ‘pilot’ studies to test whether the number of animals we have is enough for statistically meaningful data. Where possible we use archived data for re-analysis, which does not require the use of further animals. We aim to align our experiments to extract a range of data from a given animal, which further increases the power of these experiments. Finally, we have a policy to collect potentially valuable tissue from all animals where appropriate, post-mortem. This allows us to share our resources with other laboratories and act as a source for new experiments without use of 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.

We use mice in our project due to their genetic and systemic comparability to the human condition. Namely, mice share more in common genetically, behaviourally, and structurally (i.e. circuits within the brain) with humans than other lower species (e.g. zebrafish). Mice are also excellent subjects for genetic manipulation owing to their well characterised genetics. This project therefore will use mice as our animal model, with genetic manipulations as a primary method for creating models for experimentation.

Our project aims can be achieved by using behavioural tests in mice that assess the effect of various genetic and environmental alterations on mental-health associated behaviours. The majority of these tests are appetitive (involving performing simple tasks for a food reward) and provide environmental enrichment for the animals. Surgical procedures are performed under anaesthesia, and we use a common system to maintain a record of the severity of post-surgical outcomes. We use these systems to identify future refinements to our protocols to further reduce any animal suffering. The use of invasive surgical procedures is crucial to help us establish how certain genetic and environmental risk factors affect brain function in alive subjects. This increases our ability to draw conclusions about direct effects of such factors and how potential interventions may be effective.

Both the behavioural and surgical aspects of this project are collaborative between laboratories to ensure best practice is always being used to minimise animal distress and the number of animals required to achieve the project aims.

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

We are interested in effects across the life span and so a number of our animals will be maintained until old age. Less complex organisms than mice become more tangentially related to the human condition, therefore mice serve as an excellent middle ground for complexity but maintaining relevance to human physiology.

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

Example: The five-choice serial reaction time task (to measure attention) requires mice to be motivated to work for food reward, and the traditional methodology required animals to be food deprived for several months at a time. We have adopted and are piloting a new technique, were the mice have continuous access to the testing cage and are able to learn and perform the task in 5 days compared with 5 months, and because their food source is

provided by performing the task, it is only necessary to food deprive for a few days at a time, when doing probe trials.

We are also developing a new spatial maze, the appetitive dry maze, which should provide similar information to the Morris Water Maze, but without the necessity for the mice to be repeatedly exposed to water, instead they search a dry maze for a food reward.

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

We will follow LASA guidelines for Aseptic Surgery, and the PREPARE guidelines for designing experiments (https://norecopa.no/prepare).

We will also investigate and use where appropriate new open science initiatives including preregistration of studies.

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

We will use the NC3Rs website for latest information and resources (including e-learning training), user meetings and information from the Named Information Officer to stay updated with current guidance and areas of best practice.

In addition, European best practice and guidelines (via NORECOPA resources) will be applied where applicable, such as the PREPARE guidelines and NORECOPA newsletters.

We will also stay updated through the British Neuroscience Association's 'Credibility in Neuroscience' initiative and other 'open science' initiatives and adopt new methods of good practice where appropriate.

36. The Symptoms and Pathogenesis of Lung 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

Respiratory, Chronic Obstructive Pulmonary Disease, Asthma, Inflammation, Cough

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

To identify new abnormal biological processes that are involved in causing airway diseases such as asthma, chronic obstructive pulmonary disease (COPD) & idiopathic pulmonary fibrosis (IPF) and the symptom of cough. Subsequently to identify if returning these processes to there normal state will help ease the symptoms and also make patients feel better and live longer.

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

Why is it important to undertake this work?

Respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) are a significant cause of illness and death across the

world. Currently approximately 5.4 million people in the UK are currently receiving treatment for asthma, that is 1.1 million children and 4.3 million adults (Asthma UK). Although a large number of asthmatics have their symptoms controlled, many of the medications used have side effects which cause high blood pressure and increased risk of diabetes. There are also around 200,000 people in the UK who have severe asthma, this form of the disease does not respond to the usual treatments and can cause people to frequently require hospital attention. Approximately 1300 die each year in the UK from asthma.

For COPD, approximately 2% of the whole UK population – 4.5% of all people aged over 40 – live with the disease. Symptoms include a shortness of breath, a persistent cough and wheezing especially in cold weather. There is no cure for COPD and although several medications can help relieve some of the symptoms they do not stop the decrease in how well the lungs work or slow the development of the disease.

Over 6000 people are diagnosed with IPF every year in the UK and almost half of those will die within 3 years of diagnosis. Symptoms include a persistent cough and extreme tiredness, both of which are extremely distressing. There are currently no real treatments capable of halting the ongoing stiffening of the lungs or reducing the symptoms.

In healthy people coughing is protective reflex designed to expel foreign material from the lungs. However, a persistent cough, when the person has coughed for more than 8 weeks, can occur as a symptom of a number of diseases including asthma, COPD and IPF or it can have an unknown cause. Approximately 7% of the world's population suffer from a persistent cough and this symptom has a significant negative impact on the quality of the persons life. Although cough medicines are readily available from pharmacies, there is little evidence to support that they really help and therefore new and effective medicines are urgently required. If we can identify new abnormal biological processes that cause cough and then we can investigate if returning these irregular systems to normal would reduce the coughing and help the patient have a better quality of life.

It is quite likely that some of the abnormal processes that drive the respiratory diseases described above will be common across all of them. For example coughing is observed in asthmatics, COPD and IPF patients and structural changes in the airways are also common across all three of the diseases.

Many patients suffer from disease that includes aspects of both asthma and COPD. Therefore, understanding a biological pathway that is responsible for causing cough, structural changes or inflammation of the lungs may have benefits for many respiratory diseases, including those mentioned earlier.

At the time of writing this application there is a global respiratory viral pandemic resulting in severe disease and loss of life. The work described in the project will enable us to gain a greater understanding of the processes that cause respiratory diseases. This in turn should enable us to potentially target any processes which show promise with new therapeutic agents. If these agents work effectively in returning the abnormal processes to their normal state and therefore reducing the severity and/or frequency of symptoms then

data obtained will hopefully form part of the portfolio of work required to enable new medicines to be tested in human clinical trials.

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

Based on our achievements over the term of our previous project, we can expect that at the expiry of this licence we will have identified a number of new pathophysiological mechanisms involved in the most common respiratory diseases. The experiments will give raise to a significant amount of original and new information and much of this work will be presented at international conferences of professional and learned societies and then submitted for publication to peer reviewed scientific journals. We will also publish negative data and experiments where we have been unable to confirm other laboratories results with the aim of reducing unnecessary in vivo experiments. A great number of animal tissue samples will also be generated and these will be shared with other scientists and collaborators that work with us.

We also aim to assist in the progress of a number of projects and novel candidate molecules through pre-clinical research into human clinical trials.

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

The new knowledge that we will discover will be published in scientific journals and presented at conferences of learned societies. This greater understanding of respiratory diseases will enable other research groups, working in both universities and in the pharmaceutical industry, to hopefully make more informed decisions regarding their animal research and potentially lead to a reduction in numbers and also refinements.

We aim to publish our findings throughout the 5 year term of the project.

It is expected that we will be successful in progressing a number of candidate drugs through into human clinical trials. These potential new medicines may take up to 5-10 years to make it as a successful new drug. However if they are successful we expect them to have a significant positive impact on the lives of patients, their families and society as whole.

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

The new scientific knowledge that we discover will be published in books, on websites and in scientific journals and presented at both internal meetings and external international conferences. We have a large network of scientists working in universities and for pharmaceutical companies with whom we share our findings with and benefit from the discussion of our research. We are very aware that there is a need to share the findings when new medicines are not successful and we will continue to talk and discuss with other scientists and researchers in the community to share ways that will reduce the number of animals that we use and refine them so that animals will suffer less.

Species and numbers of animals expected to be used

- Mice: 14130

- Rats: 10700
- Guinea pigs: 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.

Adult mice, rats and guinea pigs are appropriate animals to study diseases of the lungs as they have many similarities in the way their lungs work when compared to humans. For example we are particularly interested in helping people who have a persistent cough and we can use guinea pigs who also cough in response to certain chemicals to help us find new medicines to treat this distressing symptom. We intend to use adult animals as their lungs are fully formed and also their immune system and circulation system is sufficiently matured and again is similar to that observed in humans.

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

A typical study may involve up to 6 groups, with 6 animals in each group. At least 7 days after delivery, animals will be given a protein, on up to 3 occasions, by either an injection of a small volume of liquid into the lungs (for this they are anesthetised) or by an injection into the cavity containing the intestines and stomach etc. A few weeks later we put the animals in a chamber and expose them to a fine mist containing the same protein. This causes them to have an allergic reaction similar to an asthma attack. Before, during and/or after this the animals would receive a number of doses of a potential new medicine either given into the lungs in a very small volume of liquid (under anaesthesia) or dosed into their stomach. By placing the animals in a special chamber to measure changes in the way they breath, where they are free to walk around, we can work out how effective our new medicine is at reducing the intensity of the asthma attack. Animals would then be returned to their home cage and closely observed to ensure they are now acting normal and not experiencing symptoms. They would be checked again at the end of the working day and then left overnight. Animals are then checked first thing in the morning and killed by an overdose of anaesthetic. We would then take blood samples and also take samples of the lung and other tissues to look for evidence that our new potential medicine has worked.

For some studies where we are trying to model diseases that take a long time to develop, such as emphysema or chronic bronchitis, the experiments may take longer. For example we know that the cause of most cases of chronic bronchitis is cigarette smoking and therefore to model this disease we expose animals to tobacco smoke over a number of weeks. This ensures that the animal model resembles chronic bronchitis as closely as possible.

More recently we have observed that some chemicals (e.g. salt solutions) that are known to cause cough in humans , do not cause cough in the guinea pig. It is important to be able to model coughing to substances like salt solutions because it is believed that these may be more relevant. We think that one reason why this might happen is that whilst humans

breath the salt solution via the mouth, guinea pigs who breath mostly through their noses, filter out the solution so that it us unable to reach the lungs and cause cough. Therefore we are performing studies where we block the noses of guinea pigs to see if they then cough to salt solutions.

We have been working on a new drug that shows great promise as a medicine for cough. However it appears that it may affect peoples ability to taste food properly. Therefore we are performing experiments investigating if the animals ability to taste sweet and sour substances is affect by the group of medicines that we are investigating.

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

Causing lung diseases such as asthma and symptoms such as cough will likely cause the animals to feel similar to a human when they have an asthma attack or an irritating cough. Typically signs that we would see in our animals would be an increase in how quickly they breath, erect fur and looking scruffy. However, these signs normally disappear completely within a few hours. Most of the studies that we conduct are short (typically less than 4 days in duration) although some experiments where we study diseases that take a long time to develop, like chronic bronchitis, could last could last for approximately twelve weeks.

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

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

For mice, rats and guinea pigs, we expect approximately 50% of animals to be classified as moderate and the remaining 50% being classified as mild or sub-threshold. This will depend on the specific studies but in many cases we will have animals who are not given a respiratory disease or are humanely killed and their tissues used for experiments. A significant number of animals who we make experience asthma or are made to cough will be treated with new experimental medicines which we expect to reduce the symptoms for them and thereby make them feel better.

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

- Killed

Replacement

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

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

Respiratory diseases occur through a complicated process involving the immune system, your genes and the environmental. The cells in our lungs, the blood vessels and the nerves and white blood cells all interact in ways which we currently cannot replicate

without using animals. This is especially true when we are trying to understand the way a potential new medicine might work.

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

Using human cells and animal cells that we grow in the laboratory and also using human tissue that we obtain from operations is an important part of our research programmes. We only use animal studies when we gather enough scientific information from those experiments to justify their use.

Why were they not suitable?

The cells and tissues that we use, although very useful, are not able to reproduce the same conditions that the human lung works in such as a working blood supply, nervous system and immune system.

We also use human lung tissue as often as we can source it, however even this is not a complete alterative to using animals as it lacks an intact circulation system and thus no white blood cells and also a complete nervous system and many other 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?

Estimates are based on the anticipated number of studies and knowing the typical numbers used in previous experiments to produce meaning full results. Over a 5 year period, animal usage may vary across protocols as the demand from as yet unknown research projects emerges. Rather than just stating the potential maximum of animals we will use across all protocols we have kept numbers within the range of recent experience so it is possible that future amendments may be required.

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

The PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines were used for planning of all the experiments using animals. The guidelines for planning animal experiments complement reporting guidelines such as ARRIVE and ensure the quality, reproducibility and translatability of animal studies, enabling the minimum number of animals are used but still producing meaning full results.

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

A significant amount of in vitro experiments, using human and animal cells and human tissue, is performed prior to any animal work and this significantly reduces animal usage. We actively source human lung tissue from both the UK and abroad and this ensures we can revise and amend animal experiments when tissue is sourced. When animals are killed at the end of a study, multiple samples of lung tissue, blood and other organs are collected and stored either deep frozen or prepared and preserved for future microscopic examination. These samples are then shared with research colleagues both within universities and with collaborating pharmaceutical companies.

Refinement

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

Which animal models and methods 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, rat and guinea pig models which enable us to study the processes involved in human lung diseases such as asthma, COPD and IPF as well as the clinical symptom of cough. We need to cause inflammation to the lungs and cause symptoms such as cough as this reflects what happens in human diseases of the lungs. It is likely that animals will experience respiratory changes and discomfort similar to those observed in patients with asthma, bronchitis and fibrosis. However, as we are seeking to find and develop new medicines, many animals would be likely to have this suffering reduced by our new treatments. We have selected airway models that are well tolerated by the mice, rats and guinea pigs and over the years we have reduced the lung inflammation that we cause to the them and thus this reduces the welfare impact on the animals, but still enables us to use them as models of lung diseases.

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

We are seeking to identify and develop new treatments for human respiratory diseases and therefore although there may be some similar biology in lower species such as flies or fish we would generally not expect potential new medicines, which have been developed using on animal experiments such as human and animal cells, to work. These lower species also do not accurately reflect the human circulation and immune systems and also the structures that they use to breath are very different. This licence application does include a protocol for purely non-recovery studies (except for compound administration) where all steps are performed under terminal anaesthesia and we use this extensively.

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

During procedures all animals are monitored continuously so that any adverse effects can be acted upon immediately. Any clinical signs that are observed normally disappear within a few hours. Animals displaying clinical signs longer than this will be assessed more frequently and additional support offered such as warming or wet mash given as needed. Advice will also be sort from either the Named Veterinary Surgeon or the Named Animal Care & Welfare Officer or both. In general animals are closely monitored and are kept in modern, purpose built facilities with highly trained and motivated technical staff. We put in place many systems to improve animal welfare, including stress-reducing handling (i.e. tunnel handling or cupping for mice), environmental enrichment such as tunnels, nesting materials and chew sticks, as well as cage floor level access to food for animals displaying reduced activity or mobility.

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

Best practice, for example the use of analgesics after surgical procedures will be employed to minimise suffering. LASA best practice guidelines will be consulted as required. We follow the PREPARE ((Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning animal experiments and we will also adhere to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) to improve the reporting of our research involving animals.

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

My team and I are in regular contact with other scientists who work with animals and with teams of animal welfare specialists across both universities and pharmaceutical companies. Members of my team regularly attend seminars and conferences organised by organisations such as the RSPCA and the NC3Rs. We also subscribe to email newsletters from the aforementioned organisations and also from professional societies such as the Royal Society of Biology, British Pharmacological Society and Institute of Animal Technology.

37. Physiopathology and therapeutic approaches for neuromuscular diseases

Project duration

5 years 0 months

Project purpose

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

Key words

neuromuscular disorders, therapeutic strategies, pathological mechanisms

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The purpose of this project is to develop therapeutic approaches (treatments) for several neuromuscular diseases such as Duchenne Muscular Dystrophy (DMD). Indeed, many of these diseases reduce life expectancy and dramatically impact the quality of life. 70,000 people have MD or related condition in UK and an estimated 7 million people worldwide. The cost per patient varies between £30,000 and

£60,000 / year depending on the disease. So far, there is no preventive or curative treatment for most of these diseases and our aim is to develop new treatments for the patients (either small molecules or viral vector coding the missing gene or allowing the expression of the altered gene (in Duchenne muscular dystrophy for instance) or inhibiting the expression of a deleterious gene (in facioscapulohumeral dystrophy)).

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

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

Findings of this project will pave the way for new treatments and pre-clinical studies for several neuromuscular diseases. They will also increase our knowledge on the malfunctioning biological processes leading to these genetic diseases. We will create new animal models because there is a critical needs for more robust animal models.

Species and numbers of animals expected to be used

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

- We use mice. Approximatively 2000 over 5 years.

Predicted harms

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

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

Some of our mouse models of neuromuscular disease develop symptoms of the disease, particularly as they get older, the major symptom being a weakness in the legs but the mice are able to walk around.

We perform surgical techniques under general anaesthesia and carefully monitor the mice postoperatively, being particularly careful to keep them warm and well-hydrated. All animals undergoing surgery will be given appropriate analgesia to minimise pain during the procedure and in the recovery period. All recovery surgery will be done aseptically. Animal undergoing local irradiation may show skin lesions (redness). Immunodeficient mice are prone to infections, so are kept in individually-ventilated cages in barrier conditions, to minimize risk of infection. We will perform treadmill running to test endurance and the experiment is stopped when a mouse remains in the fatigue zone for 5 continuous seconds. The majority of the procedures are of moderate severity. At the end of the experiments, mice will be humanely culled.

Replacement

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

Our therapeutic approaches are or have been first evaluated in vitro in a cellular model of the disease. However, as a next step towards a clinical trial, in vivo testing of potential therapeutics is unavoidable. Indeed, tissue culture is far away from recapitulating what happens in vivo, in an animal. For example, skeletal muscle stem cells do not achieve their full differentiative capacity unless they are in an innervated, vascularized setting with other cellular and non-cellular components such as connective tissue and immunological cells.

Moreover a crucial point in the development of a drug is to determine which organs are targeted and with which efficacy. In our case, we want to be sure that the muscles are massively targeted whereas other organs are spared. This cannot be assessed in vitro.

It is thus essential to evaluate our potential treatments in mice.

Reduction

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

We will use the minimum number of mice per experiment to achieve statistically-significant results and minimize the size of breeding colonies to produce the required numbers of mice. From our previous experiments we know that groups of 6-12 animals are sufficient to have statistically significant results. However, advice on the proposed experimental designs and methods of analysis of the results will be taken from a statistician. Moreover, when different experimental groups are realized, we will try to minimize the number of mice by sharing the controls across the different experiments.

Refinement

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

We use mice, as normal, immunodeficient and genetically-altered strains and models of neuromuscular conditions are available for us to perform the proposed experiments. Our breeding programme is also directed at developing animal models that reflect the condition more accurately. Animals will be closely monitored for the 1st week and every 3-4 days thereafter. As a palliative measure, dampened food may be placed on the floor of the cage post-operatively. Any animal not fully recovered from the surgical procedure within 24 hrs (eating, drinking and return to normal behaviour) will be humanely killed.

If an animal shows compromised limb function beyond 4 days after surgery, it will be killed.

All our work will be performed in the context of the emerging literature. We will take on new ideas and discard those shown to be unsuccessful.

When testing pharmacological agents, only innocuous substances would be of interest for administering to human patients suffering from muscular dystrophy.

We will obtain guidance on doses from existing studies on humans or mice and use doses shown not to have toxic side effects. If the agent being tested is likely to have any overt toxicity, a small pilot study will be performed first. Although we cannot absolutely predict toxic effects, our checking regime will ensure that any agents causing deleterious effects will be rapidly identified.

The added values of a xenograft model (in which human cells or very small human muscle biopsy are grafted into a one muscle of a mouse) are numerous. Indeed, transgenic mouse models are useful to understand the mechanisms leading to the pathology and to

develop therapeutic approaches, but they may not fully mimic the human pathology. However, to graft human muscle biopsies or cells into immunodeficient mice may better mimic the human features.

38. Understanding the womb 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

Menstruation, Implantation, Hypoxia, Inflammation, Vasculature

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 the uterus (womb) functions normally and how this changes to cause problematic menstrual bleeding or implantation disorders. Our work aims to develop preventative and therapeutic strategies for those with problematic periods and implantation 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?

Heavy menstrual bleeding (HMB) affects a quarter of women of reproductive age in the UK, i.e. approximately 4 million women. HMB has a significant negative impact on quality of life and is a huge socio-economic burden for women, their families, the NHS and society. Side effects of current medical treatments mean up to 60% of women seeking

treatment resort to risky, fertility-ending surgery. There is a clear unmet need for acceptable, effective, fertility-preserving medical treatments for HMB, yet this common, debilitating condition remains under-researched. Understanding how the uterus functions normally and what changes to cause HMB is necessary to enable development of personalised, effective medical treatments.

Up to 25% of pregnancies result in miscarriage, i.e. loss of a pregnancy before 24 weeks gestation (Practice Committee of the American Society for Reproductive Medicine, 2012). Many of these losses occur before 12 weeks and are associated with chromosomal errors in the embryo/fetus. In contrast, recurrent miscarriage occurs with normal embryos and may indicate a disorder of the womb lining (endometrium). Identification and correction of this endometrial pathology will improve the psychological and physical impact of recurrent pregnancy loss.

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

Publications: we aim to produce a minimum of four primary research papers from the work described herein.

Presentations: we aim to present these findings at scientific and public conferences, e.g. society for reproductive investigation international conference, endometriosis UK patient information days.

Clinical applications: findings from this work may inform clinical guidelines for abnormal uterine bleeding. In addition, it may identify new therapeutic targets for the treatment of period problems.

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

This project will study the normal mechanisms involved in the womb at menstruation. In addition, we will study the aberrations that lead to abnormal menstrual bleeding. Defining menstrual physiology will greatly advance our knowledge of womb function, specifically (1) the role of hypoxia (low oxygen levels) in the endometrium (womb lining) and (2) the inflammatory response at menstruation. As the endometrium repairs without scarring or loss of function during each menstrual cycle, the knowledge obtained from this project may also have more widespread benefits for other tissue sites where inflammation and scarring is problematic (e.g. lung/liver). Furthermore, these studies may identify new medications for women with problematic periods. Our models will enable proof of concept studies for new treatments. Study of the effects of diet/obesity on menstrual function will enable evidence-based lifestyle advice for women suffering from these disorders, supporting patient autonomy during clinical management.

The scientific community, patients, their relatives and society will benefit from this project. In the short term, these animal studies will benefit the scientific community due by determining how the womb works normally. In particular, the role of hypoxia in menstruation has been the source of intense debate for over 70 years. Experiments outlined here will allow us to increase or decrease hypoxia at menstruation and provide answers to this key question in reproductive biology. We will disseminate our findings

through annual presentation at national and international meetings and publication in peer reviewed journals.

More long term, it would be our intention that the outputs from this research will benefit women with problematic periods by contributing to the development of new therapies for their debilitating disorder. There is a clear unmet clinical need for new medical treatments that are effective and acceptable to women and allow surgery to be avoided. The studies performed during this project may lead to phase I clinical trials, hopefully within 5 years. A major strength of the mouse studies described herein is that they are based on our previous observational studies in human tissue, increasing the probability of translational benefits for women. Effective medical treatments for periods problems will have significant future benefits not only for those suffering from these conditions, but their families and for society. The economic cost of menstrual disorders, including surgical intervention and work absence, is significant. These mouse studies are the first step towards significant cost benefits for society.

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

Collaboration: We have strong collaborations already in place with both local and international laboratories that work on hypoxia in the lung and tumour microenvironment. These collaborations are essential for sharing knowledge, protocols, resources and expertise. This will maximise outputs from this project. In addition, we are developing a new collaboration with epidemiology colleagues, who will analyse human data to inform these studies and optimise their translational benefits. Our strong track record of studies in human endometrial tissue will inform and direct this project to ensure maximal benefits for patients.

Dissemination: We plan to present our findings at national and international conferences to ensure maximal dissemination of our findings to inform the global research effort in this important area. We have developed a patient information website, where major results from studies in menstrual function can be disseminated quickly and accurately to the public. Our research institute runs regular public lectures that have received excellent feedback. We have actively participated in these in the past and will continue to do so in the future. Unbiased publication: Our aim is to publish our findings from this project, regardless of outcome. This will inform the global research effort in this area.

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.

The human womb is important for implantation of a pregnancy. If pregnancy does not occur, menstruation (a period) occurs. Many women suffer from common, devastating

disorders of implantation and/or menstruation. Our understanding of these conditions is limited as studying human tissue disrupts the organised structure of the womb, including the blood supply and inflammatory cells, meaning interpretation of experimental results is difficult.

The mouse uterus functions in a similar manner to that of humans during pregnancy. When not pregnant, mice do not naturally menstruate. However, by administering hormones in a similar pattern to those experienced by women during a menstrual cycle, we can induce menstruation in mice. This allows the study of uterine function in health and disease without disruption of the normal uterine architecture. We only use mice greater than 6 weeks old, to make sure their reproductive system is mature enough to respond.

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

To induce menstruation in mice, we remove the ovaries of female mice under general anaesthetic before giving them estrogen for 1 week. Mice then have a progesterone implant inserted for a few days (much like a mini version of the contraceptive implant used by many women). We insert some oil through the vagina and remove the progesterone implant to trigger menstruation.

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

Mice may experience pain or infection following ovary removal or progesterone implant insertion/removal. We minimise these by giving mice a general anaesthetic for surgery and painkillers during and/or after the operation. Mice do not show any signs of distress during menstruation.

Expected severity categories and the 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 will experience moderate severities due to surgery. This is minimised by anaesthetic, painkillers and careful post operative monitoring.

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

- Killed

Replacement

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

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

Inducing menstruation in mice is necessary as the lining of the womb (endometrium) is a dynamic, hormone responsive organ made up of numerous cell types. The complex and

sophisticated control of the womb by hormones from the ovary results in changes in womb function. These include changes to the blood vessels and influx of cells from the blood, e.g. immune cells. This means that an intact uterus in a whole animal is required to carry out focused, incisive experiments to determine womb function.

These are not possible if cells or parts of tissue are removed and studied in the laboratory as tissue interactions are disturbed. Lower organisms such as flies and frogs do not have a uterus. Experiments carried out in animal models will be limited to those questions that are not possible to answer with other methods.

Previous work from our laboratory has used human cells, tissue and data analysis to generate preliminary findings on how the human womb functions in health and disease. The results of this work have allowed us to develop focused experiments using mice. For example, we have identified that the womb becomes hypoxic (low oxygen levels) during menstruation in women with normal periods.

Women with heavy periods appear to lack this menstrual hypoxia. Therefore, we seek to manipulate the hypoxia system genetically and pharmacologically to determine impact on menstrual function.

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

Hysterectomy specimens/womb biopsies

Human womb cells

Why were they not suitable?

Hysterectomy specimens/womb biopsies from women with objectively measured heavy and normal menstrual bleeding. These samples have been used to identify differences in the womb between those with heavy and normal periods. However, the endometrial architecture is disturbed with tissue removal making study of blood vessels and immune cell influx very difficult. In addition, these samples cannot be used to see if these differences are a cause or consequence of heavy menstrual bleeding. In addition, it is difficult to collect the large numbers of samples required to normalise for the inevitable variability between different women.

Study of human womb cells. This will be used where possible, but does not allow the study of the interactions between different cell types and influx of cells from outside the womb.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 estimated based on the number of mice required in our experimental and comparator groups to examine the impact of hypoxia, inflammation and obesity on womb function. These numbers are required to provide meaningful results to inform future development of preventative and therapeutic strategies for women with problematic menstruation.

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

We have searched the available literature and on line databases to ensure our experiments do not unnecessarily duplicate published work.

We have arranged sharing of resources with other researchers and close control of our breeding strategies to ensure minimal animal use.

Statistical advice has been sought to increase the precision of our experimental data and limit the number of animals used. Careful experimental design will limit numbers of mice used, utilising 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?

We will use efficient breeding strategies to optimise the number of animals used. Pilot studies will be performed where appropriate to optimise experimental protocols. Our carefully managed mouse tissue database facilitates reuse of the mouse tissue collected for multiple laboratory experiments, reducing 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.

Mice do not naturally menstruate but we can simulate menstruation in mice with a protocol optimised in our laboratory to closely resemble the changes seen in the human womb. Mice will be used in our experiments as they are amenable to pharmacological and genetic manipulation with the preservation of cell and tissue structure. Mice will be at least 6 weeks old to ensure appropriate reproductive maturity and decrease unsuccessful induction of menstruation.

All surgery will be performed with aseptic technique, appropriate anaesthesia (e.g. isofluorane in 100% oxygen) and analgesia given routinely at the time of surgery. Post-operative analgesia will be administered as required (buprenorphine 0.05-0.1mg/kg s.c. or in diet). In our experience, surgeries are well tolerated with a recovery time of less than an hour. Post-surgical complications are unusual but we will monitor mice closely post-operatively to aid early detection and treatment. We will maintain close relationships with the NVS/NACWO to continually refine our behavioural and health monitoring procedures.

Mice will be housed in groups, including following surgery. Group numbers will be in line with the code of practice. Appropriate environmental enrichment will be supplied.

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

Lower organisms, such as flies and frogs, do not have a uterus. Menstruation is limited to women, old world primates, fruit bats and the elephant shrew. Simulated menstruation in mice limits experimentation on primates, such as the rhesus macaque.

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

Menstruation is a feature of normal reproductive function in women and higher primates. A mouse model of simulated menses was reported in 1984 and has undergone subsequent refinement.

Introduction of oil to the womb via the vagina/cervix has been established in our laboratory and this is used wherever possible to reduce the number of surgeries.

For implantation studies, we will aim to optimise a transcervical method of embryo transfer to reduce the number of surgeries required in our experimental design.

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

Where more refined approaches become available during the course of this licence either through personal communication, publications or veterinary advice, we will investigate their use following discussion with the NVS and Home Office Inspector.

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

Where more refined approaches become available during the course of this licence through personal communication, publications or veterinary advice, we will investigate their use following discussion with the NVS and Home Office Inspector.

39. Innate antimicrobials in defence against infection

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Infection, Virus, Bacteria, Antimicrobial, Immunology

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

This project aims to understand the mechanisms by which antimicrobials, produced naturally by mammals, can prevent and combat infections and the harmful responses to infection.

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

Why is it important to undertake this work?

Infectious diseases are a major cause of death and illness around the world. The growing problems of antimicrobial resistant bacteria, and harmful viruses for which we have no vaccine or effective treatments, demonstrate the need for new approaches to prevent and treat infections. Understanding the most effective mechanisms by which antimicrobials, produced naturally by mammals, can effectively combat infectious diseases and modify the body's responses to them, will help to develop urgently required new therapeutic and preventative strategies.

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

This project is expected to lead to a range of outputs, including advancement of scientific knowledge with clear translational potential to impact upon novel interventions for infectious diseases, publications, presentations, funding applications, public engagement resources/events and press releases as appropriate.

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

Infectious diseases are a major cause of morbidity and mortality. Despite a golden era of antibiotics, respiratory infections alone account for 1 in 15 deaths in the UK, and ~18% of mortality in children under 5 years of age globally. Bacterial pathogens resistant to antibiotics now represent an ever increasing challenge, prompting international fears of an “antibiotic apocalypse”. In addition, new viruses, and the recently demonstrated ease of their rapid global spread, represent a threat for which vaccine development can be too slow, and effective therapeutics for viral infections are rare. Finding new approaches is an urgent international priority.

Targeting the most effective components of our naturally-occurring defence systems, promises new preventative and therapeutic approaches for infectious and inflammatory diseases. However, a greater understanding of these natural protective mechanisms is required; from microbicidal properties to the mechanisms of modulation of immune responses, the role of the microbiome, and the effects of aging.

The short term benefits of this project will be to advance our mechanistic understanding of Host Defence Peptides in protection against infection/inflammation. This advancement of scientific knowledge, with clear translational potential to impact upon novel interventions for infectious diseases, is expected within a 5 year period. Successful discoveries, with the potential to lead to the develop of new treatments (most likely to be for viral lung infections) could then be developed over the following 5 - 10 years.

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

The impacts and outputs of this research will be maximised by early dissemination, publication and presentation of findings in open access formats, and by optimising local and international collaborative approaches (including with biotech industry if appropriate) and data sharing to advance the work in the most rapid and effective manner.

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.

To complement studies performed with cells in the laboratory, and human clinical studies, mice will be used; studied at neonatal, adult and aged Life stages to evaluate the Life

stages at which specific infections are found to occur in humans, and related to well known changes in the immune systems of mice and humans with age. Mice have been shown to closely share key features of the immune system with humans, and to be good models for understanding effective responses to infection in the lungs, using the microbes of interest in this proposal. In particular, their natural "antimicrobial host defence peptides" (the major system studied by this proposal), are very similar to humans. The availability of mice in which these defences have been genetically altered, and demonstration that this leads to less effective defence against these lung infections, makes this the ideal animal in which to understand how new approaches can be developed, targeting these systems, in humans.

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

Typically mice will sniff a small droplet into their lungs, to introduce a viral infection, and will also inhale substances designed to boost their defences to that infection (up to once a day, typically for one week). Some of these mice will be the offspring of genetically-modified mice, with specific elements of their defence to infection targeted to work less well. The mice will be observed and weighed, before being humanely killed to study the effectiveness of the infection and the treatments.

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

The main impacts expected in mice with lung infections are weight loss and some decreased activity, typically over one week. Where a small minority of mice have more substantial responses to the early phases of infection, they may have loss of body temperature, significantly decreased activity and shortness of breath for a few hours maximum.

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

The majority of animals (~75%) used on this licence are expected to be subthreshold, used only for breeding of genetically-modified mice with not harmful characteristics. Of the remaining 25% of animals, the majority (~70% of these) are expected to have only mild signs, associated with mild infections that are well controlled, cleared and boosted by treatments, with the remaining (estimated at less than 30%) having experiences of moderate severity (principally in relation to weight loss).

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

- Humanely killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 body's natural defences to infection, and then using that information to develop new approaches to preventing and treating infections, is of ever greater global importance. Projects, such as this, with those aims, need to combine studies using the best approaches to studying cells in the laboratory, with clinical studies characterising the outcomes in humans. However, that combination still cannot provide all the answers and needs to be complemented with studies in animals. These studies allow the researchers to examine all the biological components of the response to infection, and to actively modify it, from the initiation to the recovery, in a living system that is greater than the sum of its parts.

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

This project has considered, and is undertaking, studies using bacteria and viruses alone, studies using cells grown in the laboratory, studies using materials from humans (both healthy volunteers and infected patients), and studies characterising relevant aspects of human health and responses to naturally occurring infections, as alternatives to the use of animals, to address some of the key components of this project.

Why were they not suitable?

Studies using bacteria and viruses alone, studies using cells grown in the laboratory, studies using materials from humans (both healthy volunteers and infected patients), and studies characterising relevant aspects of human health and responses to naturally occurring infections, are all suitable to answer some the questions required to meet our aims. However, testing the hypotheses that we can create from these studies ultimately also requires the use of live animals. These complementary studies in animals then enable researchers to modify the biological responses to an active infection (to block and/or boost them) at different defined stages and observe the consequences for protection against infection in all the multitude of components of a living system that is great then the sum of its parts. This is not possible to do using non-animal alternatives alone and is essential for the development of new preventative and therapeutic approaches to infection.

Reduction

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

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

The number of mice necessary for this project have been estimated based both on records from previous related research, to determine the scale of breeding colonies required, and using mathematical approaches to estimate the numbers of mice required for individual experiments to provide reliable levels of data.

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

Extensive use of studies using cells grown in the laboratory, and use of human samples, to precede and complement studies using animals, means that mouse numbers used in experiments will be significantly reduced.

In designing experiments for this project, approaches have included consulting experts in experimental design and statistics, using experimental design training from FRAME (an independent charity, working to promote alternatives to animal testing), and using the NC3R's (National Centre for the replacement, refinement and reduction of animals in Research) 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?

In addition to good experimental design, this project will optimise the number of animals required by using efficient breeding protocols, small, targeted pilot studies to focus experimental approaches, approaches that maximise the number of scientific questions that than can addressed in each animal, and via the sharing of 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.

This project will use models of lung infections of particular global health importance, such as using viruses for which we have no effective treatment or vaccine, or bacteria with high levels of antibiotic resistance, for which new preventative and therapeutic approaches are urgently needed. Lung infections will be initiated and allowed to develop in the least harmful manner possible while still causing targetable natural defence systems to be activated; achieved by using well documented previous studies, pilot studies, careful clinical monitoring and appropriate further responsive-mode refinement of approaches. At different phases of disease, from initiation to recovery, researchers will intervene to boost or to block the natural defence systems to clearly identify promising interventions. This may be through administration of drugs or cells, or the use of genetically-modified mice (in which host defences are automatically disrupted without further active procedures or harmful characteristics). Suffering during periods of infection will be minimised by using the lowest dose of infection and the shortest time course of infection possible while still make valid use of the study to answer the specific questions, in addition to support measures (such as active maintenance of body temperature) to minimise the severity of symptoms

that cannot be avoided. In any cases where clinical monitoring identifies an unexpectedly higher level of severity, the experimental animal will be humanely killed.

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

Animals that are less sentient (such as drosophila and zebrafish) can be used to examine certain aspects of defence against infection. However, for these specific studies, mice are the least sentient animals in which all the major components of the natural defences against infection are characterised as sufficiently similar to those of human to enable valid evaluations of their relative importance and interplay within a living organism. This level of complexity is required at this stage in the experimental scientific research and development of novel preventative and therapeutic approaches for future human use.

Conducting infection time-course studies over the required timeframe cannot be conducted under terminal anesthesia.

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

To refine the procedures being used, to minimise the harm to animals, the research team have developed and use a clinical scoring system for infected animals. This is used hourly in acute phase of infections, and daily in less severe, longer infection periods, to ensure that harm is minimal, and to enable further refinement of protocols where any deviation is observed, particularly when using animals that are potentially more susceptible than usual. Using this system, the approaches can be refined to use lung infections that develop in the least harmful manner possible while still causing targetable natural defence systems to be activated, and over the shortest periods possible to answer the specific questions. Additional refinements, already in place, include support measures, such as active maintenance of body temperature, during the early acute phase of bacterial infection, developed to minimise the severity of responses to infection.

Our team, in particular animal technicians (who can conduct, train and supervise procedures), are experienced in handling and conducting experimental work on animals, which will reduce stress and suffering of the animals. The unit in which the animals are kept is large, well-resourced and well- equipped. Throughout all work, advice is sought from the animal technicians and from veterinary staff. Every experiment is submitted first to a vet for review, advice and scrutiny of procedures. Prior to and during experiments, animal health will be maintained using good breeding and handling techniques and housing in individually ventilated cages.

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

The ARRIVE 2.0 guidelines will be used to ensure that experiments are conducted and reported in a valid and appropriate manner, and the Experimental Design Assistant from the National Centre for the replacement, refinement and reduction of Animals in Research (NC3Rs) will be used to ensure the most refined experimental design.

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

The team will stay informed about the 3Rs during this project, and implement appropriate advances. This will be achieved by reading relevant publications, and attending appropriate presentations / conferences. In addition to those disseminating the relevant scientific advances, these will include information from the local Animal Welfare Ethical Review Body, local Annual Refresher Training, local annual 3Rs day of presentations, and output from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)

40. Presynaptic function and dysfunction in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain cells, Epilepsy, Neurodevelopmental Disorders, Neurodegeneration, Therapy

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

To determine the role of presynaptic function and dysfunction in health and disease.

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

Why is it important to undertake this work?

It is important since an understanding of how the brain cells communicate in both health and disease is critical to sustain efficient neuronal communication if it is disrupted. Many brain molecules have poorly defined functions, and many communication processes are not understood at the molecular level.

Furthermore, how these processes contribute to higher brain function is still unclear. The work outlined in this project addresses these points, and will use the information acquired to understand how dysfunction in brain communication culminates in human disease. By determining where dysfunction exists at multiple levels of complexity, and critically, how this dysfunction may be corrected, is of the highest urgency and importance.

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

The output from this programme of work will primarily provide new information, specifically a more sophisticated and accurate understanding of the key molecular steps that underpin brain communication. This is important for two major reasons 1) an in-depth knowledge of the function of specific molecules required for brain communication will allow a focused study of genes (and mutations in these genes) identified as being linked or causal to human disease and 2) a determination of which aspects of brain communication are disrupted in models of human disease. Importantly, our research strategy allows the convergence of a future therapeutic programme of work at the level of brain processes, rather than individual molecules. This should allow a greater diversity of interventions in pathways that occur either in parallel to, or are integrated with, the affected brain processes.

Another output is the generation of new models of human disease. A demonstration that the disruption of specific neuronal processes can translate into an easily measurable outcome in animals will be of high value for researchers in academic and commercial settings. This is because it will increase the possibility of translating progress from basic to applied research, by providing a means to screen for future drugs using simple measures.

The aim is that by the end of this 5 year project we will have identified key targets for future intervention in a number of neurodevelopmental disorders and neurodegenerative conditions and may have identified potential new drugs to combat these disorders.

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

Scientists studying brain communication will benefit, since this group will be able to exploit this new knowledge in their own research. This may provide novel and hitherto unexplored avenues of research for a number of academic and/or research groups.

Research funders such as UK Research Councils and various charities devoted to the treatment of neurodevelopmental disorders and neurodegenerative conditions will benefit from the results of this project as it will validate the value of the scientific research that they support.

Other beneficiaries will be companies that investigate disorders of the brain, such as autism, intellectual disability, epilepsy and neurodegenerative conditions. The identification of specific molecules and processes required for correct brain communication and critically interventions that can correct brain dysfunction, will provide new drug targets and therapeutic interventions respectively. The benefits to this group will be longer term, however if specific drugs can be used that are already approved for human use, this could allow drug trails in companies to commence immediately.

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

The results from this work will primarily be reported in scientific publications in specialist journals. It will also be presented at international conferences focussed on brain communication and human neuronal disorders. Furthermore, the work will be publicised to a wider lay audience through scientific communication events and via social networking sites such as Twitter.

Importantly, we will also publish the outcomes of unsuccessful approaches, in terms of failure of specific interventions to correct observed defects in brain communication, and also where testing of specific hypothesises regarding such communication were disproven.

If outputs indicate that the project would benefit from collaboration with other scientific groups or companies that possess specific expertise, this would be enthusiastically followed.

Research funders will publicise the outputs, especially high-impact work, since this provides proof of benefit of their financial support.

Species and numbers of animals expected to be used

- Mice: 16250
- Rats: 7250

Predicted harms

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

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

Rodents are the most appropriate species for our work, not only because they are amenable to genetic manipulation, but studies have identified that the key molecules that will be the subject of investigation are highly conserved between both rodents and humans. The great majority of the work outlined will be performed using tissue from embryonic or neonatal rodents. Adult rodents will only be used in specific instances to obtain brain tissue to investigate the composition or circuitry of specific brain region. The only time living rodents will be used in a procedure will be to assess their seizure susceptibility or the impact of their aging. These experiments are essential, since they allow the effect of drug interventions to be tested.

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

As stated above, the vast majority of animals will be culled simply to utilise their brain tissue. Culling will be either by schedule 1 procedures or other procedures that are occasionally required to maintain the functionality of the tissue. A small fraction of experiments (less than 5%) will involve the delivery of virus particles to the rodent brain via surgery and injection. Another fraction (less than 5%) will be performed in living animals, this will involve on occasion the delivery of drugs either orally or via injection. The seizure susceptibility experiments are very brief (lasting 2 minutes), whereas the aging studies, by definition, last longer. If any animal displays distress for these two procedures they will be immediately culled.

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

There are no expected adverse effects in any of the genetically modified rodents we plan to use. The adverse effects arising from surgery and injection of virus particles will be minimised by best practice in pre and postoperative care and appropriate analgesia. In seizure susceptibility experiments rodents may progress through to full seizure within the 2 minute experiential period. These rodents will be humanely culled immediately. Rodents that are part of an aging study will be closely monitored for adverse effects such as weight loss or abnormal gait. Animals that continue to lose weight will be humanely culled.

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

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

The expected severity for the majority of the procedures under this licence is anticipated to be sub- threshold (approximately 80 %). Breeding and maintenance of genetically-modified rodents will occasionally reach mild severity (5 %). Culling for CNS tissue (approx 5%) and aging studies (approx

2.5 %) will be mild. Both seizure susceptibility to audiogenic stimuli (2.5 %) and surgery and delivery of virus (5 %) 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?

This project studies the role of molecules in brain communication and how alterations in this communication result in neurological disease. For this study an experimental system of appropriate complexity must be used in relation to the cross-talk within individual brain cells, how they communicate with other brain cells and ultimately how this translates into altered behaviour. The only appropriate experimental system that allows the study of such processes through increasing complexity are vertebrate animals.

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

As the field currently stands there is no alternative to using brain cells from animals. We investigated the possibility of using "transformed" cell lines to mimic the communication inside brain cells. These cells can grow and multiply on their own in a dish, negating the requirement for animal brain cells.

We also investigated establishing measurements of brain cell function in stem cells taken from patients with neurodevelopmental / neurodegenerative disorders. These can be induced to behave in a manner similar to mammalian brain cells.

Why were they not suitable?

Transformed cell lines cannot currently mimic the intricate series of molecular events observed in brain cells. In most cases they also require a series of interventions to make them “neuronal”. Therefore it is unlikely that this system will faithfully reproduce the events that occurring inside human brain cells.

Human stem cells that are induced to appear "neuronal" are a promising avenue for the future. However as the field currently stands, these cells appear to be highly "immature" and do not produce the same molecules or communication pathways currently observed in brain cells taken from embryonic rodents. Therefore the ability to investigate the complex communication within and between mammalian brain cells is still not 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?

Power analysis calculations will determine how many animals will be used for specific experiments. These calculations will be guided using established statistical methods that we have applied to these types of data over the past 10 years, and that have been subject to scrutiny during peer-review. Brain cells grown in culture are a reproducible system, meaning we can reduce the number of animals required to reach statistical significance (usually less than 6 animals). This number is slightly higher for experiments using brain tissue (normally 8–10 animals are required for each experiment). Also there is a chance that altering the genetic makeup of the animal may lead to greater variance in a particular phenotype. Power analysis calculations can be performed to accurately estimate the number of animals required to complete the aims with statistical significance in this regard.

For experiments that require observation of animal behaviour, these may require the use separate experimental groups (i.e., control minus drug, genetically-altered minus drug, control with drug, genetically altered with drug). Previous Power analyses suggest that group sizes of at least 15-20 will likely be necessary.

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. As such, genetically altered animals (or experiments using brain tissue) will always be tested alongside unaltered wild type animals (or brain tissue) that are matched for age and gender, and treated groups will always be compared to vehicle controls. Furthermore, experiments will be performed blind to genetic makeup by using a code (e.g., A or B) to identify each animal. This code will be kept by a person who is not involved in the experiment. Groups will be assigned in a randomised fashion.

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

Cultures of brain cells will be shared among the research group to permit as many experiments as possible to be performed per animal. This is also the case for experiments using brain tissue.

Researchers can and often do use material from one animal for multiple experiments. In many cases, two researchers prepare tissue from the same animal to perform their experiments.

Finally, all of the systems and behavioural experiments in the application are extremely well characterised by both ourselves and other research groups, meaning that almost all animals will be utilised in a productive manner. Wherever possible both male and female animals will be used in our experiments, again reducing the number of unnecessary deaths.

Refinement

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

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

Rodents are the most appropriate species for our work. One reason is that they are amenable to genetic manipulation and numerous studies have identified that the key molecules we are investigating are very similar in both rodents and humans. Furthermore some aspects of the research programme require investigation of region-specific effects in mammalian brain (e,g, striatum loss in Huntington's Disease). Therefore having an experimental system that is built in the same general manner to humans is key to this programme. Also, genetically modified rodents accurately recapitulate the genetic disease state in individual brain cells, providing a refinement not possible with other alternatives, such as destroying brain regions.

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

Cultures of brain cells (where the majority of the work is focussed) are derived from rodent embryos or pups within their first week of life. Therefore we are already using an immature life stage for this work. The study of brain circuits requires greater complexity, therefore brain tissue has to be obtained from older rodents. Animal behaviour has to be observed in conscious rodents by definition, therefore there is no alternative in this instance. Less sentient species are not appropriate for these studies, since we require the complex behaviours observed in rodent models.

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

In most cases culling of animals will be performed using schedule 1 approaches. The genetically modified (GM) mice we plan to use initially display only mild phenotypes. For any known age-dependent adverse effects of genetic makeup animals will be culled before any distress is known to occur. Novel GM animals that may become the subject of investigation will have their health monitored closely.

Where required, principles of good practice for surgery will be followed (e.g. establishment guidelines for 'Anaesthesia in Rodents' and 'Surgical Procedures on Experimental Rodents'). Pain will be controlled during surgery by general anaesthesia and pre- and post-surgery by analgesics. Risk of infection will be minimised by good surgical and aseptic techniques. Surgical sites will monitored for signs of inflammation and infection.

For audiogenic seizure experiments, we will follow published methodology but with important refinements. In the published methodology seizure is graded on a 4 point scale (wild running, clonic seizure, tonic seizure and death). In our protocol we will test only one

GM animal at a time (paired with a control); this will allow us to stop the experiment and humanely kill an animal should a tonic seizure occur. Thus, we will assess audiogenic seizure on a 3 point (rather than a 4 point scale) in order that death is not an endpoint of the seizure. In order to compare our data with that of other laboratories who have conducted such experiments we need to carry out this protocol on awake animals to assess any behaviour that the auditory stimulus initiates – using anaesthetized animals and recording brain activity, for example, is not possible with our proposed protocol nor is it practical in terms of the age of animals being studied.

Another further refinement is the expertise and experience of care and research staff within the laboratory and animal facility. Rodents will have access to a well-resourced and well-equipped modern facility with individually ventilated cages and barrier systems in use for maintaining “Specific Pathogen-Free” (SPF) status/health. Where appropriate to animal health and wellbeing colonies will be provided with environmental enrichment.

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

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 visit the 3Rs webpage regularly to keep abreast of the latest developments. Myself and my research group will also attend 3Rs seminars and webinars where available. Where an obvious advance in 3Rs is apparent, we will move to quickly adapt our protocols to conform with the new guidance.

41. Investigating cardiovascular disease using the 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
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

zebrafish, Cardiovascular, Heart, Regeneration

Animal types	Life stages
Zebra fish	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?

Identifying novel molecular pathways that regulate cardiomyocyte proliferation and regeneration following 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?

There are millions of people living and dying due to heart and circulatory diseases in the UK every year. While survival rates are improving, an ageing population will ultimately lead to more heart disease in the future. Loss of heart tissue can result from common conditions such as heart attacks or high blood pressure leading to decreased heart function and heart failure. Replacing lost heart tissue could restore heart function in damaged hearts. One potential way to achieve this would be to stimulate the heart to regrow new tissue. However, while the zebrafish can do this, the human heart cannot. This project seeks to better understand the specific molecular pathways and mechanisms that permit the zebrafish to achieve this and to explore whether they can be harnessed for human benefit.

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

All work will be published in peer reviewed journals in a timely fashion. Data will also be used in grant applications to generate further funding for ongoing research.

We anticipate a future grant to then take current projects forward into mouse regenerative models and provide further evidence in humans and understand the importance of underlying regenerative molecular mechanisms in CM vs other cell types.

The findings are expected to lead to novel pathways that could be targeted for drug treatments for human cardiovascular disease.

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

In the short term, 3-8 years, new pathways and mechanisms will be elucidated. This will benefit of the scientific community investigating potential molecular pathways that play a role in myocardial regeneration. In the longer term, possibly 10 or more years, nitrosylated proteins and LncRNA-Carmen could be of benefit in animal models such that both molecules could be targeted as candidates for novel treatments for heart disease.

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

The work involves collaborations with other molecular scientists across the University campus and internationally. The work will be shared with key funding bodies such as the BHF and MRC and also shared across various publication and web platforms thus ensuring a wide distribution and sharing of the knowledge and ideas arising from the proposed work.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 7000

Predicted harms

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

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

The zebrafish is unique in that it can regrow new heart muscle tissue following injury. Studying this will allow us to seek new mechanisms that might allow human hearts to undergo a similar form of self-repair after a heart attack for example. The adult zebrafish, 90-120 days after fertilisation will be used as this is the key time when the zebrafish heart has a strong capacity to regrow.

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

The adult zebrafish will undergo an operation to cause heart injury either by surgically removing a small portion of the heart muscle or injury caused by freezing a small portion of the heart using liquid nitrogen. Both types of injury will be done by an experienced operator under a full general anaesthetic. The animal will then recover from the operation for a period of up to 60 days before being killed for investigation. We add morphine to the bathing water routinely in the post-operative period to address pain relief.

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

The animals recover from the operation fairly quickly, typically within 24 hours. There is occasionally some bleeding from the wound. The fish start swimming around the fish tank within 4-6 hours after surgery and appear to show no major adverse effects. Fish are monitored throughout the surgery and the 24 hour period thereafter and euthanased if showing signs of distress or are unlikely to recover. Despite this we still find that we have a 5% mortality within the first 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)?

Both types of heart injury operations using either surgical resection or cryo-injury represent moderate severity, any animals showing severe distress during the operation are not allowed to recover from the initial general anaesthesia and are euthanased using an overdose of anaesthetic.

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

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 culture experiments cannot provide the in-situ, mixed-cell, whole organ environment required to study cardiac regeneration. The zebrafish whole heart regeneration model permits the study of multicellular tissue incorporating intrinsic tissue remodelling and an acute and inflammatory response to injury all of which can be studied in this model and could not be readily studied in other replacement models such as tissue culture. We do however perform similar experiments in larvae at much earlier developmental stages whenever possible to minimise suffering to older adult fish.

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

Tissue culture models of cardiomyocytes have been considered but these are limited by the issues highlighted in the section above. Larval models of heart injury have been extensively used in my laboratory but the immature heart does not contain the same complex mixture of cell types such as fibroblasts, mature immune cells and vascular smooth muscle cells that are present in the regenerating adult heart.

Why were they not suitable?

Adult cardiomyocytes do not grow well in in tissue culture and the mixed cellular components, and their associated extracellular milieu, cannot be replicated in tissue culture setting. In addition, as described above, the adult zebrafish heart produces a modest fibrotic scar in response to cryo-injury which does not arise in the larval animal and is difficult to reproduce in tissue culture. This scar formation creates a model that is very similar to that found in mammalian model systems and in humans after myocardial infarction. This makes the adult zebrafish models described in this application highly relevant for human disease.

Reduction

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

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

These estimated numbers take into account a set of experiments from two specific, funded projects addressing key issues regarding the molecular pathways of cardiac regeneration. The numbers are based on previous experience in our Lab and in other labs in our institute. The numbers are also based on the protocols described in each of the funded projects. However, the majority of the adult fish used under this license will be utilised for breeding and maintenance which has a maximal severity limit of mild.

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

We used a number of approaches including - advice from our local responsible veterinary officer, the NC3R's design assistant and we took advice from other colleagues performing similar experiments using zebrafish.

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 share tissue across different members of our lab group and other members of our centre. We have a very pro-active team in our aquatic facility that monitors and streamlines all fish lines.

Refinement

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

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

Two Adult Zebrafish heart injury models will be used in this project: heart resection and heart cryoinjury. Most of the experiments will be done using the cryoinjury model which is technically less demanding, and which produces a myocardial scar similar to that seen in mammalian models of myocardial infarction. In our experience of using these models, once recovered from surgery the vast majority of animals survive to the end of protocol at 60 days. Suffering is minimised by carefully observing them in the immediate 2 hours following surgery, allowing them to recover in a dark area, if they show any signs of distress based on monitoring protocol and scoring system then they are killed quickly by overdose of anaesthetic. Once they are over this 2 hour post-surgery time point, we typically observe good recovery with severity not exceeding moderate.

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

We do currently use a model of heart laser injury in larval zebrafish but this model has limitations from a scientific viewpoint in that the heart is very immature, the cellular response to injury is driven mostly by usual-embryonic growth and the heart is very small and thus much more difficult to extract, stain and analyse histologically.

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

All members of my research group will continue to seek new ways to minimise harm caused to our animals by attending regular, appropriate training courses, liaising closely with our local animal care staff and veterinary services, reading appropriate literature and remaining vigilant for the welfare of our animals on a day to day basis by maintaining good communication and relations with colleagues in our aquatic facility.

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

We will use online material at zfin: https://zfin.org/zf_info/zfbook/cont.html

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

We will remain continuously engaged with updates from NCR3's via email updates and web-posts, we will continue to maintain and develop our training and skills with regard to animal welfare. We will remain engaged fully with our local animal care staff and veterinary services.

42. Breeding and Maintenance of Genetically Altered Rodents

Project duration

5 years 0 months

Project purpose

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

Key words

Breeding , Genetically altered

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

Retrospective assessment

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

Objectives and benefits

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

What's the aim of this project?

The aim of this project is to create, breed and maintain mice with genetic alterations and supply them to investigators to carry out research into the study of biological and physiological processes and/or the control of disease.

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

Why is it important to undertake this work?

Genetically altered (GA) rodents are widely used in the fields of biological, medical and veterinary science, and are considered to be of great value in dissecting the function of genes and pathways in physiological and pathological processes. GA animals also provide us with models for many human diseases.

The application of GA mice is well established at our establishment to study biological and physiological processes and/or the control of disease. The data generated from these studies are essential to provide new knowledge at the cellular and molecular level, and to validate new prophylactic or therapeutic approaches for disease.

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

This project aims to breed, maintain and supply GA mice to our research teams and to those at other establishments working in collaboration with us. The outputs at the end of the project will be in the form of new information and publications from the investigators and research groups that we have provided animals to.

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

Scientists working on projects using GA animals at our establishment will all benefit from an efficient supply of animals for their research. Outputs and advancements in these scientific areas will be published and benefit the wider scientific community and advance medical knowledge. Where practical, animals will also be available for collaborators at other institutions. Overall, this may result in less numbers of animals bred and used.

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

Many Project Licence holders collaborate with other establishments in many research areas and publish scientific data and findings observed throughout the studies conducted.

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.

This project aims to breed, maintain and supply adult GA mice to our research teams and to those at other establishments working in collaboration with us.

The types of mice bred and maintained will be determined by our Project Licence holders' requirements for their Home Office-approved research projects.

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

Mice will be placed in pairs or trios and bred to produce GA animals.

For certain breeding schemes, a small tissue sample (drop of blood or ear notch) may be collected at weaning to examine if the offspring is of the correct GA status.

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

There are no adverse effects expected for the animals in this project, as the GA strains bred under this licence are not expected to show any harmful phenotypes.

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

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

Mild severity for 100% of the animals is expected

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 aim of this project is to breed, maintain and supply GA mice to investigators to carry out Home office-approved research into the study of biological and physiological processes and/or the control of disease.

To date, there are no in vitro assays that adequately model the molecular, cellular and physiological interactions that take place in an intact animal. Nor is it possible to perform studies in which immune/pathological responses to infection of human subjects are manipulated or examined in a controlled manner. Therefore it is necessary to use a whole-animal model.

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?

Each Home Office-authorized Project Licence at our establishment has numbers of animals to be used specified in their licence. These animal numbers are added up, and this figure is used to determine how many animals will be produced on the GA production licence.

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

Animals will only be bred if a user requirement has been established, and the breeding programme will be subject to regular review to optimise production in line with anticipated demand. Breeding will be optimised, wherever possible, to produce only the genotype required.

Unnecessary breeding of GA animals will be avoided by searching cryobanks and databases (e.g. from the National Centre for the replacement, refinement and reduction of Animals in Research, the Jackson Laboratory, and the Federation of International Mouse Resources).

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 the “traffic light system” for all our mouse breedings. This is a standardised system to determine the quality and efficiency of breeding colonies.

Refinement

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

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

The purpose of this project is to breed, maintain and supply GA mice to other investigators. Animals are not expected to display pain, suffering or distress.

Access to the breeding room is limited to ensure animals are housed in quiet areas with minimal disturbance.

For some mice, a small sample of tissue (drop of blood or ear notch) will be taken at weaning for genotyping to examine the genetic status. Published guidelines for current best practice will be followed to minimise animal suffering

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

N/A

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

Breeding animals are checked twice daily by a competent person. Breeding colonies are maintained by staff who are licensed and trained to the highest standard, including how to recognize any signs of ill health, pain or distress.

All animals are handled using the cupping or tunnelling technique in order to minimise stress.

All animals bred under this licence are subject to a rolling programme of environmental enrichment to ensure they do not exhibit any adverse effects due to boredom and lack of stimulation. This enrichment programme includes, tubes, tunnels, mouse houses, sunflower seeds and other available enrichment item

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

The "Advice on Severity Assessment of GA animals" published in 2014 will be used, along with published articles from the NC3Rs, Understanding Animal Research, and RSPCA on GA animal breeding.

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

The licence holder keeps up to date with any advancements in the industry. The licence holder is also a member of a number of networks that share best practice and advances in the 3Rs, and regularly and actively seeks out new information and updates regarding breeding practices. This knowledge is shared with staff involved in mouse colony care and maintenance.

43. Discovery of new 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
- 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, Metastasis, Pharmacokinetics, Therapeutic efficacy

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?

The work performed under this licence aims to develop novel agents that can then be assessed clinically as potential treatments for 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?

Around one in four of all deaths in the UK are caused by cancer, highlighting the urgent need for improved cancer treatments. Many novel molecularly-targeted anti-cancer agents have been developed, and some show significant benefit in subsets of cancer patients.

However, the duration of drug-induced tumour responses is often short-lived due to the emergence of drug resistance, and for many patients there is currently no beneficial treatment.

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

This project will contribute to evaluation and progression towards clinical trials of potential novel anti- cancer agents targeting key features of cancer such as metastasis, resistance to standard of care drugs, drugs, regrowth after regression/treatment, that are unresolved issues in current cancer treatment and the cause of many cancer-driven fatalities.

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

Ultimately, cancer patients may benefit from these outputs if drug candidates progress to clinical trials. Deeper biological understanding of cancer biology will be acquired and therapeutic hypotheses will be confirmed or refined based on this work.

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

We have close collaborations with colleagues from the Establishment and we collaborate with them to discover inhibitors for their targets and assess our inhibitors in vivo in their models. The results of this work will be published and/or disseminated through presentations at scientific conferences when appropriate and will therefore inform other cancer researchers worldwide.

Species and numbers of animals expected to be used

- Mice: 10650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 development is dependent not only on the changes occurring within the transformed cells, but also on the interactions of the cells with their microenvironment. Mice are more comparable to humans than less sentient model systems (fish, invertebrates) in pathophysiology and show higher levels of conservation in nucleotide and amino acid sequences. This is important as we intend to use reagents such as small molecule inhibitors and antibodies that have been developed to target human proteins.

While cell-based research has elucidated many changes in cancer cells, it provides little information about the local environmental factors influencing early-stage cancer development in life. Also certain hallmarks of cancer, such as spread of cancer and blood vessel formation, are impossible to study in vitro (cell culture). Therefore, mouse models are important for studying the in life aspects of human cancer development. Mouse models have been engineered to develop tissue-specific cancers, which accurately mimic their human counterparts, and have potential applications to test the effectiveness of novel

cancer therapeutics. Moreover, non-protected species and less sentient species do not have specific organs such as lungs, so we would be unable to use them for animal models of lung cancer involving the injection of cancer cells in the organ where they are found in man.

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

Normal and genetically altered mice will be used to investigate the efficacy of modulating certain specific molecular pathways to suppress induction, progression and metastatic spread of cancer cells.

Most mice used, will experience only administration of agents by either mouth or injection into a vein, under the skin or in the abdomen and taking very small blood samples as we determine the concentration of drugs (up to 24hr) and tolerability studies (up to 28 days) with gradual increments in doses given.

The mice may have tumours grown in them initiated through either use of cancer prone genetically modified mice or by the implantation / injection of existing tumour cells / tumour pieces. Surgical procedures requiring general anaesthesia and pain relief are tumour implantation (most commonly subcutaneously) and tumour biopsy (a gold standard method used clinically).

A combination of methods will be used to monitor tumour growth: the use of calipers (for subcutaneous implanted tumours) and imaging under light general anaesthesia will be performed on a number of occasions. The effect of potential new therapeutics upon tumour growth will be investigated either alone or in combination with existing agents to determine their potential future utility in patients.

On occasions it will also be necessary to inject, by one of several possible different routes, chemical agents that will allow better imaging of the internal tumours. The majority of animals are not expected to show signs of adverse effects that impact on their general well-being. Very rarely the severity of these signs may be such that the humane end points may be reached (i.e. 20% loss in bodyweight) and the mice killed humanely. The majority of the procedures will result in no more than transient discomfort and no lasting harm.

All the mice 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?

Novel agents that will be used may not have a history of being used in mouse models. There is potential therefore for acute adverse effects of intolerability. The body weight and other signs of discomfort will be monitored daily; transient weight-loss is common with many anti-cancer therapeutics. Mice will be observed more frequently during the first two hours after novel agent administration.

Lethality resulting from drug toxicity is anticipated to be very rare; furthermore, in our experience not more than 2% of mice should experience a level of toxicity that

necessitates humane culling of the animal. Regular observations of the animals should ensure wherever possible that mice are culled prior to drug-induced deaths.

The impact of tumourigenic mutations are not expected to cause any adverse effects per se as these in most cases only manifest following administration of inducing agents. It is possible that the tumour growth might affect normal physiological functions (such as eating, locomotion or breathing). However, mice will be observed daily and any side effect that cannot be managed satisfactorily will result in humane culling of the animal.

Injections would only cause very transient pain.

After surgical procedures we will monitor mice for signs of pain and administer effective pain relief for as long as it is required.

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

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of mice used in drug exposure / tolerability studies are expected to experience no clinical symptoms due to the administration of novel agents before they are humanely killed. We will use very small does and only two animals per group initially when the first administration of a new agent is used. Additionally, some mice will experience the discomfort of repeated (daily) injections of therapeutic agents or oral delivery with a specialist tube. We will aim to utilise the least stressful route of administration wherever possible.

The vast majority of mice used in tumour / efficacy studies are only expected to experience the mildest clinical symptoms due to tumour growth before they are humanely culled. Some mice may experience a degree of skin ulceration. Dry ulceration is considered a sign of healing and are not considered harmful. Mice that develop ulcers that impact adversely on their well-being will be killed humanely.

A minority of mice will undergo surgery and these will be anaesthetised for the operation and receive pain killer post-operatively until pain subsides. Some mice will also have repeated anaesthesia for the purposes of imaging the internal tumours. Whilst loss of consciousness may be distressing this is not painful.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are necessary to test concepts in tissue settings under physiological conditions that resemble those in humans. Treatment of organisms with anti-cancer agents induces complex responses involving interactions between different types of tissues and organs.

In order to test the activity of cancer drugs, it is necessary to use models of cancer grown in an appropriate environment. Immuno-incompetent (nude or SCID) mice are routinely used for these studies because they can support the growth of human cancer cells as a non-mouse tumour.

Furthermore, in this proposal, we will examine the effects of drugs on the subcutaneous growth of human tumours and this can only be achieved in a complete organism that has a fully functional circulatory system.

Additionally, it is a legal requirement that any drug for human cancer use is assessed in at least one mammalian species before entry into clinical trials. Finally, mice are also routinely utilised in cancer research for the assessment of drug exposure, tolerability and therapeutic efficacy.

The research plan follows well recognised international guidelines, which aim to facilitate and accelerate the development of anticancer pharmaceuticals and protect patients from unnecessary adverse effects, whilst avoiding unnecessary use of animals, in accordance with the 3R principles (reduce/refine/replace).

Which non-animal alternatives did you consider for use in this project?

- Mammalian cell cultures.

Why were they not suitable?

These responses cannot be recapitulated using in vitro methods alone or with simple organisms (e.g., Mammalian cell cultures) that lack the differentiated system of an animal. Multiple pathways that together determine the fate of a drug in human, such as adsorption, metabolism, distribution, excretion, and pharmacological and toxicologic effects cannot be mimicked by an in vitro system. It is established that treatments in humans have comparable biological and biochemical activities in animals.

Conclusions from animal experiments are therefore relevant in understanding human disease and 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 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.

Data available from the literature or from pilot studies are used to perform power analysis to determine an appropriate sample size for the definitive experiment. In general, we will use a sample size capable of detecting a 40% practical difference with 80% power and 95% confidence.

Based on past experience, group sizes of between 6 and 12 animals (dependent on the readout) per experimental group suffice. However, we may use several doses of a drug, or several different drugs or treatment combinations to test a hypothesis. The number of experiments and groups per experiment is estimated based on the stage of the projects and the estimated number of compounds that following progression through the screening cascade will be appropriate for in vivo evaluation. This is based on our experience of bringing multiple drug discovery projects from hit identification to candidate nomination and clinical trials.

Considering power, the number of experimental groups, and the number of drug targets we are interested in, we have then estimated the total number of mice to be used over the licence lifetime.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior to any large scale experimentation, initial pilot studies will be conducted to characterise models. This will allow us to better estimate take rates after cell implantation, observe recovery rates from surgery, and observe how the mice cope with the tumour burden and any clinical observations that may indicate complications. Further standard experimental procedures will also be assessed for applicability, for example, the measurement of tumour growth, inter- and intra-tumour variability, and the measurement technique employed. The information gathered will help to establish the numbers needed to power larger studies for statistical significance, in parallel with the best processes for the monitoring and care for the animals during these experiments. Experiments are designed using the principles outlined in the experimental design tool on https://eda.nc3rs.org.uk/ and reported according to ARRIVE guidelines. Power analyses will be performed, ideally with the support of a biostatistician or mathematician, to ensure that we use the minimum number of mice to generate statistically validated results.

As a result of advances in blood biochemistry analysis (e.g. highly sensitive high performance liquid chromatography mass spectrometry HPLCMS/MS technology coupled with ‘micro-capillary bleeds’), it is possible to analyse circulating drug levels from very small blood samples (~10µL blood volume). The small sample volume allows serial samples to be collected from the same animal, thereby reducing the total number of animals required for pharmacokinetic studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Use of animals will be minimised by (i) making use of in vitro model systems (e.g. tumour cells in the presence of drugs) to test specific hypothesis wherever scientifically justified, (ii) use of in vivo bioimaging to follow disease development and response in real time (rather than culling cohorts of mice at defined time points), (iii) the use of pilot experiments to test for the extent of an expected phenotype prior to a full scale confirmatory experiment (thus avoiding full scale experiments that may lack sufficient statistical power), and (iv) making use of a screening cascade to limit the number of compounds and therefore the number of mice required for the in vivo investigation stage.

Experiments will be appropriately controlled and mice of the same age, genetic background and source used to reduce the variability of results and to produce highly consistent data. Wherever possible and appropriate, a single group of animals will serve as a control for duplicate experimental group. The proposed experimental designs and methods of analysis of the results will follow statistical guidelines and involve discussion with our bioinformatician or mathematician scientist to provide sufficiently powered studies, minimizing the number of animals used in each experiment. The design of individual experiments will generally involve factorial designs, which maximise the information obtained from the minimum resource.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 humans share significant similarities in genome structure and gene complexity, in comparison with other less sentient model systems. Moreover, the mouse is well understood and is an adequate model to use for this work. Previous studies have used the mouse, thus use of this model avoids the need to repeat earlier studies in a new species.

Initial pilot studies and consultation with industry experts will aid in identifying the ideal mouse strain as well as to characterise the model, as detailed above. Experience within the Establishment with PD studies has helped refine treatment scheduling. This has led to a reduction in animal numbers and improved inter-animal variability. Such investigations and improvements are continuous within the institute and will be implemented where appropriate.

As a result of advances in blood biochemistry analysis (e.g. highly sensitive HPLCMS/MS technology), it is possible to analyse drug levels from very small blood samples. The small sample volume is a refinement, because warming time prior to sampling can be reduced as well as quicker/simpler sampling method reducing procedural handling. The use of these serial micro-sampling methods has been implemented for all dose

escalation/toleration studies and provides additional exposure data without the need to run an additional study. In mice we usually use 6 animals per route, due to limitations in blood volume that can be taken, with a staggered blood sampling over an 8 hours period. This is also a refinement to the traditional method of using an animal per sample time point, and reducing inter-animal variability, providing 3 values per time-point for improved statistical significance whilst greatly reducing the total number of mice required per study.

Targeting pro-drug delivery to specific tumours offers a Refinement for the systemic exposure of active compound since the conversion from the administered pro-drug is concentrated within the tumour cells expressing the viral transfection.

Why can’t you use animals that are less sentient?

Only a mammalian lung cancer model system has the potential to accurately mimic both the anatomy and complex cell biology, including microenvironmental interactions, of human normal and tumoural lung. Furthermore, there is considerable experience in the wider scientific community regarding the use of mice as a model system for human malignancies and many reagents exist for the phenotypic characterisation of mouse cells.

Mice are the lowest sentient species that are appropriate for in vivo drug development studies and are widely used for this purpose. Most of our work is carried out using well-characterised human tumour cells, grown in the appropriate anatomical site in naturally immunodeficient adult mice to avoid tissue rejection. This enables us to study human cancers in the correct tissue microenvironment.

How will you refine the procedures you’re using to minimise the welfare costs (harms) for the animals?

Suffering is minimised by keeping tumour sizes within tolerable and acceptable limits and according to recognised guidelines. Compounds are delivered using previously determined well-tolerated doses and schedules, and are generally of low toxicity (e.g. agents targeted to molecules selectively overexpressed or mutated in human cancers). We will make the most of non-invasive methods such as optical imaging, bioluminescence, CT-Scan, radiography, optoacoustic, ultrasound.

The conditions under which the experimental animals are kept and used for the proposed procedures are designed for the least possible disruption of natural behaviour and quality of life. The animals are maintained in individually ventilated cages using sterile food and bedding with enrichment of the cage environment and all procedures are carried out in special cabinets using strict aseptic techniques to avoid infections. We constantly work to improve husbandry and procedures to minimize actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare in situations where the use of animals is unavoidable. As detailed in each protocol, there is provision for the appropriate anaesthetic and analgesic regimes as well as appropriate culling methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555-77 (2010)] administration of substances will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.

For monitoring the aged mice we will follow the guidelines of Wilkinson et al (2020) Laboratory Animals, Vol. 54(3) 225–238.

Guidelines for Body condition score. [Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23].

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 / activities locally. Through discussing refinements with our NACWO, NVS and HO inspector..

44. Mapping and controlling neural circuits

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuroengineering, Neuroscience

Animal types	Life stages
Mice	adult, embryo, pregnant, juvenile, 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 main aim is to develop new tools and techniques that help us understand how the brain is altered in disease and help to correct those alterations. Ultimately, these tools can eventually be applied to diagnose and treat 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?

The most important discoveries that have been made recently in neuroscience have relied on new tools for analyzing and controlling the nervous system. For example, most of the neuroscientists at our institution rely on optogenetics, a system for controlling the activity of brain cells, in order to understand the effects that individual brain cells have on animal

behavior. Most neuroscientists also rely on the use of specialized proteins called calcium indicators, which can tell neuroscientists when individual brain cells are active. These tools (calcium indicators and optogenetics) were both developed by neuroengineers like myself. In contrast to most areas of neuroscience, neuroengineers do not typically seek to understand how the brain works, but instead seek to build tools that will help other neuroscientists understand how the brain works. The tools that we develop lay the groundwork for the next generation of neuroscience.

In all cases, we thoroughly test new tools in cultured cells or in computational systems before using them on animals. However, our goal is to develop tools that can be used by neuroscientists to better understand the mammalian brain, or that can be used in humans for therapeutic purposes. For that reason, it is critically important that new tools be tested on animals to determine whether or not they work for their intended purpose. The protocols described in this license application are intended to allow us to test new tools in animals, before deploying them widely for other neurosciencists to use.

In the long run, the experiments we are doing will have a major impact on neuroscience and the treatment of neuropsychiatric disease. Some of the tools we are developing will enable neuroscientists to study the brain at a higher level of detail, revealing new aspects of how diseases affect the brain. In addition, we are also developing tools for controlling brain activity, which may eventually be used directly for therapeutic purposes. For example, we are exploring new ways to diagnose neurodegenerative diseases such as Alzheimer's disease, and are building new gene therapies for debilitating psychiatric and neurological disorders such as epilepsy. The use of animal models allows us to evaluate the safety and efficacy of these tools before they would ever be used in humans.

What outputs do you think you will see at the end of this project?

This project will advance our understanding of how networks of neurons are organized within the brain, how cell types are distributed throughout the brain, and how neuronal activity is organized within the brain. Moreover, this project will produce new technologies aimed at controlling the organization and activity of brains. This knowledge will be disseminated through presentations at scientific conferences and peer-reviewed publications. In addition, any new techniques, reagents, or software tools generated as a part of this project that have potential to impact clinical or broader scientific practice will be disseminated either freely, through partnership with an appropriate company, or through commercialization in a startup company.

Who or what will benefit from these outputs, and how?

Our goal is to produce new technologies that lead to major advances in the diagnosis and treatment of brain disorders, including psychological disorders such as schizophrenia and depression; neurological disorders such as epilepsy; and neurodegenerative disorders such as Parkinson's and Alzheimer's. We will achieve impact in these areas both directly, through the creation of technologies that can be applied for therapeutic purposes in humans; and indirectly, through the creation of technologies that allow researchers to better understand the effect of these diseases on the brain, thereby allowing them to develop newer therapies.

The methods we develop with direct therapeutic potential will include new techniques for delivering gene therapies to the brain as a way of producing gene-replacement therapies for debilitating diseases such as Fragile X. In addition, we will produce new methods for inhibiting gene expression in specific cell-types, for example to allow us to knockdown expression of genes that cause disease such as the Huntingtin gene in Huntington's disease.

On the other hand, some of the indirect methods we will generate will include methods for characterizing brain circuits and the distribution of cell types throughout the brain, thus allowing us to observe how tissues are affected by disease.

Our goal is ultimately to make our technologies available as widely as possible, so scientists, doctors, and patients can all benefit from them.

How will you look to maximise the outputs of this work?

Whenever possible, we will seek to maximise the utility of data and tools generated as a part of this project through collaboration with other experimental or theoretical groups, and online distribution of raw data and software tools. We will also make use of preprint platforms, such as bioRxiv, to rapidly disseminate our findings to a broad audience.

Species and numbers of animals expected to be used

- Mice: 27150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

We are developing many different kinds of tools, all aimed at recording or manipulating different properties of the nervous system. The overall organization of the brain and the function of cells in the brain is similar across mammals. Therefore, insights gained through studying the effect of molecular tools on the structure and function of the mouse brain will help understand what effects these tools would have on the human brain. In addition, the availability of genetically altered mice makes it possible to measure and manipulate neural circuits with unprecedented precision.

Most experiments will be conducted in adult mice. In the case of some tools that are aimed at recording properties of the nervous system during development, or that are aimed at manipulating neural development (e.g., gene therapies that could be used as interventions for neurodevelopmental disorders), we will use neonatal animals. In addition, mouse embryos or oocytes will be used when required for the development and maintenance of genetically altered mouse lines.

Typically, what will be done to an animal used in your project?

Typically, animals will be subjected to a single surgery under suitable anesthesia and analgesia with post-operative follow-up. During the surgery, a molecular tool we have developed (such as a gene therapy) will be injected into the brain, but these tools are not expected to have adverse effects. Animals will typically then be allowed to recover for an extended period of time, unless they exhibit adverse effects requiring early termination, or must be terminated early for scientific reasons. Rarely, animals will be subjected to a second procedure, under appropriate anesthetic and analgesia, in which a chemical will be injected to stimulate neural activity or changes in neural connectivity. Finally, animals will be terminated and tissue will be collected for further investigation.

What are the expected impacts and/or adverse effects for the animals during your project?

Pain resulting from surgical procedures may reach moderate severity for short periods of time immediately following surgery. Animals will be closely monitored for signs of pain after surgery and appropriate analgesia will be provided.

Head fixation is expected to result in only mild stress during initial habituation to the experimental apparatus.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

16350 mice at mild severity. 10800 mice at 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 are still far from understanding the effects of different neuroscientific research tools on the brain. For example, we have previously tried to develop research tools in cell culture, and have found that the levels of gene expression in cultured cells can be very different from the levels of gene expression in the mouse brain. Many other features of biology cannot be satisfactorily replicated in in vitro systems: for example, the blood-brain-barrier is a major barrier to the development of brain therapies, and despite several advances there are not yet satisfactory models for the blood-brain-barrier in vitro. Since our ultimate goal is to create tools that can be used to develop new therapies and diagnostics for use in humans, it is important that we use model systems that are as close as possible to human biology, hence the need for animals.

We will focus on mice, which are genetically tractable allowing highly precise measurements and manipulations of neural circuits and the development of disease models, and whose brains are organized according to similar principles to humans.

Which non-animal alternatives did you consider for use in this project?

Computer simulations Brain organoids

Stem-cell-derived model systems

Why were they not suitable?

Often, when tools fail, they fail because of some element of the underlying biology that is not yet understood. Elements of biology that are not understood cannot be modeled in computer simulations. This is why, for example, even simple organisms such as worms and flies still cannot be simulated in the computer faithfully.

Organoids can reproduce many of the aspects of the brain, but still differ in ways that are important and often unknown. Moreover, brain organoids lack normal sensory inputs or outputs, and as a result the connectivity and activity of neural networks present in organoids may differ substantially from the networks present in animal brains.

Finally, stem-cell-derived model systems are excellent for studying aspects of cellular biology, and we expect to use such systems widely. However, they are not effective for studying aspects of the nervous system such as connectivity or activity for the same reason as organoids.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals are estimated based on our prior experience breeding genetically altered mouse lines and with experimental approaches used in the project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will always ensure that we use the minimum number of animals possible to achieve our research goals. We will conduct pilot studies and adopt new methods as they become available, to ensure that we are always extracting the most information possible from any particular experiment or animal. In addition, we will develop better analysis methods and experimental design to ensure that we achieve maximal power and minimal variability.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will always conduct pilot studies to test the efficiency and applicability of new tools and techniques before employing them broadly to pursue the scientific objectives of the project. We will use computer modeling and first-principles modeling to refine engineering designs and hypotheses. Whenever possible, brain tissue and experimental data will be shared between researchers or will be used for multiple experiments or 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.

This project will exclusively use mice. Surgical procedures will be conducted under deep anaesthesia following aseptic technique and mice will be carefully monitored following surgery to ensure complete and uneventful recovery.

Why can’t you use animals that are less sentient?

For projects that have direct clinical application, it is very important to use models that are as close to humans as possible: for example, the adeno-associated viruses that we are experimenting with only infect mammals, and previous studies involving adeno-associated viruses have been found to be invalid because they were used in animals that were not sufficiently similar to humans. Carrying out experiments in this project solely in terminally anaesthetized animals is not feasible since many of our experiments require gene expression, which takes place over the course of several weeks.

How will you refine the procedures you’re using to minimise the welfare costs (harms) for the animals?

We will ensure always to use appropriate analgesia and anesthesia, with close monitoring following surgery. We will habituate mice gradually to any new apparatus in order to minimize stress, for example in the case of head fixation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines set out in LASA Guiding Principles on Preparing for and Undertaking Aseptic Surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will utilize online resources such as the NC3RS and RSPCA, and will work with the staff of the biological research facility, such as the NIO, NACWO, and NVS, in order to improve and refine our procedures and adopt new techniques as they become available.

45. Tumour targeting of novel anti-cancer immunotherapies

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cell-based therapy, nanomedicine, immunotherapy, Cancer, macrophages

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?

This project involves the testing of novel cancer immunotherapies alone or in combination with traditional cancer treatments in preclinical mouse models of 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?

Curing cancer is one of the big challenges of the 21st century. The global cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018. Our knowledge of cancer has greatly improved, and this has revealed the huge variability that can be found between not only different types of cancer, but also between patients with the same type of cancer. Cancer is responsible for over one quarter of deaths in the UK. Many conventional drugs like chemotherapy and radiotherapy are good at killing cancer cells, but they also kill normal healthy cells. New targeted therapies are urgently needed. Immunotherapies that activate the immune system to identify and attack tumours have now become a clinically validated treatment for some cancers. The research we are undertaking seeks to improve the tumour targeting of immunotherapies alone or in combination with current cancer treatments. If successful this could make a big difference in the way cancer is treated, taking us closer to being able to 'cure' this disease.

What outputs do you think you will see at the end of this project?

The major outcome of this work will be identifying a clinical strategy for translation of novel cancer therapeutics into patients and will directly influence phase I clinical trials as a mono or combination therapy. This work will be published in frontline journals and will provide valuable insights and new information about these targets and their role in cancer. Once we have published our findings and protected our new drugs (e.g. through patents) the drugs developed in this work programme will be shared with other researchers who think they may be useful for the treatment of other diseases.

Who or what will benefit from these outputs, and how?

In the short-term, this work will benefit the scientific community by providing valuable knowledge on new targeted drugs for cancer and the response of the immune system to these drugs. It is likely that the drugs investigated here will also be useful in other cancer types, and the novel compounds will be made available to other researchers for further exploration.

In the mid-long term, the ultimate benefit of this work will be to cancer patients. During this project we also predict sensitivity and resistance to the compounds, for identification of patients who are likely to respond to the treatment.

How will you look to maximise the outputs of this work?

We will collaborate with industry and academic partners for the safety and toxicity testing of these drugs, who are better equipped to perform these experiments. This will streamline the process of taking new drugs through pre-clinical testing, allowing us to more quickly move forward promising compounds into clinical trials. We will disseminate our findings at conferences and in frontline journals and where possible include negative data in these.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

We will use mouse models of cancer as these are well established, reproducible and reliably mimic much of the disease in patients. Many studies will use human tumour cells, to most reliably replicate the human disease. However, implantation of human tumour cells must be done in mice with a deficient immune system. For some studies it will be important to have a fully functioning immune system, for example for drugs targeting the immune system, in this case mouse tumours will be used.

For each experiment, the model used will be guided by studies in cells and patient screening. In each circumstance, the advantages and disadvantages of each mouse model will be weighed and the model that most appropriately addresses the experimental aims, while adhering to the 3Rs, will be used.

Where possible we will use tumour cells genetically altered to allow specialised imaging of tumours that allows for tracking of tumour growth over time, to reduce the number of mice needed in each study. We will use mice of an appropriate age for each model that allows for effective engraftment and tumour growth, this is typically a young adult mouse.

We will perform as many experiments as possible in cells and/or organ cultures and screen patient samples (where possible) before we do any experiments in animals. Many cancer therapies are highly effective at killing cancer cells when treated in cell culture, but not as effective when treating a whole animal. This is because of the complex environments and signalling networks within animals that may prevent the therapy from working, as yet this cannot be modelled in a dish or by a computer. For example, sometimes drugs do not get through the body to the cancer very effectively, or the body might remove the drug before it can do its job.

We will use cell/organoid culture and screening of patient samples to identify sensitive cancer types and effective drug combinations. We will combine this with computer modelling to understand as much as possible about how the drugs work before treating animals, e.g. how drug resistance may develop. Studies in mice will only be performed

after this to show that the treatments are effective in a whole animal model, and also to identify which new or approved drugs should be used in combination.

Studies will be designed in line with the NCRI Guidelines for the welfare and use of animals in cancer research (Workman et al. 2010). Throughout the study we will re-evaluate the models used in line withthe 3Rs (replace, reduce, refine), to ensure we are using the best and most ethical animal models and where possible use non-whole animal models.

Typically, what will be done to an animal used in your project?

Some animals will be injected or implanted with cancer cells. Where possible, this will be done in the way that most accurately mimics with disease in patients (e.g. injected into the blood for blood cancers, or into mammary tissue for breast cancer), sometimes this will require a surgery under general anaesthesia. In some cases, tumour cells will be injected under the skin as this provides easy access and monitoring of the tumour and avoids complex surgical procedures.

Some animals may be fed a modified diet for a part or throughout the study as this can improve how effective some therapies are in reducing tumour. Some animals will be fed or injected with compounds that we are developing to treat cancer, or a placebo, this is usually once a day or up to 3 times a week. Animals may also be treated with other cancer therapies including radiotherapy. Animals will be weighed at appropriate intervals to monitor weight loss, as this is a sign that they are unwell.

To monitor tumour, animals will be put under general anaesthesia for specialised imaging, this is usually once a week. We will also take a small amount of blood from a vein in the tail once a week to measure markers of response which can be detected in blood. For some tumours, other specialised techniques may be used to monitor the disease. For example, for cancers which cause bone disease we will use micro-computed tomography to scan an affected bone under general anaesthesia. or if magnetic therapies are administered we may use MRI to track delivery as well as monitor tumour size.

The duration of each study will depend on the model used, studies using more aggressive tumours may only last 3 weeks, whereas less aggressive tumours can develop over several months. Experiments to study the effect of compounds on normal biology without tumours present, will last for a defined treatment period, usually this will not be more than 2 months. Humane endpoints will be used to define the end of each study, such as tumour size, detection of relapse or metastasis, or after a period of treatment.

Animals will be humanely killed at the end of the study or when they show signs of becoming significantly unwell, whichever is first.

What are the expected impacts and/or adverse effects for the animals during your project?

Occasionally some mice will experience adverse effects, but provisions will be made to minimise these. For example, the cancer may make some animals feel sick and they may stop eating or drinking resulting in weight loss, their activity may change, or they may look

scruffy. We will weigh and check the mice regularly to carefully monitor for signs that they may be unwell. We will also measure the tumour regularly, and not allow the tumours to grow beyond reasonable limits. If the mice lose too much weight and don't recover within 24 hours, seem unwell or if their tumour gets too large, they will be humanely killed.

When blood samples are taken, if too much blood is taken the mice can become anaemic. To make sure we do not take too much blood mice will be weighed and only an appropriate amount of blood willbe taken from each mouse on each occasion (https://www.nc3rs.org.uk/mouse-decision-tree-blood- sampling).

The drugs and compounds we will treat the mice with are not expected to have any adverse effects at the doses we will use. The injection or feeding of these drugs will not cause any more than a short discomfort at the time of administration.

General anaesthesia for imaging or tumour implantation is not expected to have any adverse effects. Mice will be closely monitored during anaesthesia and if breathing becomes irregular then 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)?

Animals will experience a maximum of a moderate severity level. It is expected that 99% of animals will experience a moderate severity, some animals in which tumours fail to grow will only 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?

Our overarching goal is to develop new treatment combinations for cancer. To achieve our aim of validating novel compounds for the treatment of cancer, and find effective therapeutic combinations, we will take a range of approaches. We will perform experiments in 2D and 3D (e.g. spheroid and organoid) cell cultures, fluid flow systems, perform screening in primary patient samples, use computer modelling of large complex data sets and use mouse models of cancer. While non-animal models of cancer are very helpful in understanding aspects of biology and response to therapeutics, unfortunately they do not as yet model the response of a whole organism. However, as much as possible we will replace animal models with non-animal models and we will continue to investigate non-animal alternatives throughout the duration of this licence.

We will perform as many experiments as possible in 2D and/or 3D cell cultures and where possible screen patient samples before we do experiments in mice. Many cancer therapies are highly effective at killing cancer cells when treated in cell culture, but not as effective when treating a whole animal. This is because of the complex environments and signalling networks within animals that may prevent thetherapy from working, as yet this cannot be modelled entirely in a dish or by a computer. For example, sometimes drugs do not get through the body to the cancer very effectively, or the body might remove the drug before it can do its job.

To understand cancer biology and therapeutic response, mice provide reliable and robust models. To study both human and mouse tumours, we will use mouse models of cancer to validate our findings in non-animal models in a whole organism model of cancer. Findings from these studies will then influence translation to clinical trials.

Which non-animal alternatives did you consider for use in this project?

Animal experiments are one key part of this work programme required to achieve our aims, which we will also combine with non-animal models.

We will use 2D and 3D (e.g. spheroid and organoid) cell culture and fluid flow models to study the biology of new targets and to test novel compounds. This will include understanding tumour response and the mechanisms that govern these responses. By screening in cells from a wide range of cancers we will be able to identify sensitive cancer types and potentially biomarkers that predict sensitivity or resistance. We will also test our compounds in combination with other potential cancer therapies, including novel therapies and those already in the clinic. The most promising cancer indications, and drug combinations will be validated in mouse models. We will also screen primary patient samples with our compounds to identify sensitive cancer types. Finally, some experiments performed in cells will generate large sets of data. To understand this data in a meaningful way we will use computer modelling. This will help us understand why some cancers are sensitive to our compounds and why some cancers have existing resistance or develop resistance after prolonged treatment.

Throughout the study we will continue to investigate non-animal alternatives through resources such as the NC3Rs. Alternatives will be implemented when relevant.

Why were they not suitable?

The non-animal experiments are all suitable models for early investigation of new drugs for cancer treatment, and we will use these to complement studies in mice. However, experiments in a dish or computer are unfortunately not capable of reproducing the effects in a whole organism e.g. do not have a complete vascular system to study drug delivery.

Many cancer therapies are highly effective at killing cancer cells when treated in cell culture, but either do not work or are less effective when treating a whole animal. A whole animal has many natural barriers that can prevent a drug from working the way it would on an individual cell. After entering the body, the drug may never reach the tumour or the drug may be metabolised and removed from too quickly to have an effect. Even if the drug

reaches the tumour, the tumour microenvironment for most cancers is much more diverse than that which can be modelled in a dish, and this can also influence drug responses. Finally, there can also be systemic effects that may change the response to the drug.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used quantitative data from previous studies for statistical analysis as well as online tools and specialised software to estimate the numbers of animals we will use. In particular we have used the NC3Rs Experimental Design Assistant, PREPARE guidelines (Norecopa) and G*Power software.

Where appropriate, we will perform pilot studies on small numbers of mice to determine the optimal number of mice for each cancer model to use the lowest number of mice required to achieve statistical power

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have planned to use a number of non-animal models (e.g. cell models and screening of patient samples) prior to experiments in animals to reduc the number of animals required.

Experiments were planned using the NC3Rs Experimental Design Assistant and the Norecopa PREPARE guidelines, G* Power Software was used for power calculations and experiments were planning in line with the National Cancer Research Institute's Guidelines for the welfare and use of animals in cancer research (Workman et al. 2010 Brit J Cancer).

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 we will use pilot studies to identify optimal compound doses and mouse numbers to use for each model. We will continue to use the NC3Rs Experimental Design Assistant, Norecopa PREPARE guidelines and G* Power Software. For complex experiments, we will seek advice from specialist statisticians to help in experimental design.

We will use mouse models of cancer as these reliably and reproducibly mimic many aspects of tumour growth and metastasis in humans and can be manipulated for use of specialised techniques, like tumour imaging. We will also use inbred mice because this minimises variation between mice, thereby reducing the number of animals required.

Where possible we will use labelled tumour cells that allow tracking of tumour over time with specialised tumour imaging. We will also use other specialised monitoring techniques for specific cancers, e.g. micro computed tomography for cancers that cause bone disease. This will reduce the number of mice required for experiments, by performing measurements over time in the same animal rather than requiring multiple groups to investigate different timepoints.

In some instances, we may use multiple treatment groups with one control group. For instance, if we want to test more than one compound in one cancer model and the compounds can be administered on the same schedule. This will reduce the number of studies and hence reduce the number of animals used.

Where possible we will store tissue that may be of interest to investigate in the future, even if analysis is not planned in the original design. This will minimise the risk of having to repeat a study to obtain more tissue/data. We will also make this tissue available to other researchers upon reasonable 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 use two general types of mouse models of cancer to study the effects of new potential cancer therapies. The first uses human tumour cells or tissue implanted into mice that lack part of their immune system. This is required so that the body does not reject the human tumour cells. The second model, uses mouse tumours implanted into mice with a fully functioning immune system. This is also important as the immune system can influence tumour growth and response to therapy. These models have been extensively used by us and others, so there is a wealth on information available on these models and they are considered to be reliable and reproducible. We will plan experiments to not let tumours grow to a size where they cause the animal to become significantly unwell, we will use earlier timepoints at which the animals will be humanely killed.

To minimise harm to animals, we previously developed refined mouse models of prostate cancer, that have minimised harm, stress, suffering and pain to mice by identifying earlier endpoints that to not rely on the animals showing signs of being unwell. This was made possible through specialised imaging techniques that can detect the disease at much earlier stages. This also made monitoring therapeutic intervention more accurate and allowed further refinement of one model to mimic a specific disease state that occurs in many myeloma patients. This is something that we implement as much as possible in models of other types of cancer. We will use specialised, non-invasive tumour imaging

techniques under anaesthesia, which allow us to identify and implement study endpoints that do not rely on animals showing signs of being sick, in distress or in pain, while also reducing the numbers of mice required.

Only animals deemed to be in good health will be used in experiments. Animals with a compromised immune system will be housed in ventilated cages through a biosecurity barrier to minimise their risk of infection. Any techniques that may be painful or distressing to animals will be performed with anaesthesia and appropriate pain relief will be given to minimise pain (e.g. after surgery). All surgeries will be performed under sterile conditions following best practice guidelines (e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery). Animals will be regularly monitored for possible adverse effects that may occur in response to the tumour, procedures or treatments. We will seek advice from the NVS and/or NACWO where appropriate (e.g. for administration of an antibiotic if an infection is suspected. Optimal treatment regimens (e.g. dose, frequency and route of administration) will be identified in small pilot studies before use in larger scale studies.

We will also use general practices that minimise stress, harm and pain to animals. This includes non- aversive methods for handling mice (e.g. not picking up by the tail), the use of single-use needles to avoid pain from dulled needles and NC3R guidelines on blood sampling.

Why can’t you use animals that are less sentient?

To study effect of treatments on tumour growth, metastasis and relapse requires a whole organism model. Mouse models have been extensively used by us and others, so there is a wealth on information available on these models and they are considered to be reliable and reproducible. Mice are also very similar to humans genetically, and many processes in relation to development and growth of cancer are conserved between these species. Mice that lack part of the immune system can also grow human tumours, allowing us to study the effect of new treatments on human tumours.

Some less-sentient species, such as zebrafish, can be used to study specific tumorigenic processes. However, these models cannot be used for studying the effects on intervention on processes like metastasis. They also do not provide the ability to model treatment strategies that are used for patients. For our studies, in order to determine whether our new treatments are effective at treating growth of primary tumours and/or metastasis, mice are the most reliable model and we cannot currently perform these experiments in a less-sentient species. We find mouse models of cancer are most reliable and representative in adult mice and they cannot be used at a life stage where mice are less-sentient.

How will you refine the procedures you’re using to minimise the welfare costs (harms) for the animals?

Tumours will be regularly monitored through non-invasive methods. Mice will also be weighed to check for signs of weight loss that may occur in response to the tumour or treatments. Mice will be regularly checked for signs of being unwell, for example changes

to the coat condition, behaviour and movement. We will also continually attempt to identify earlier endpoints for studies, to minimise harm to animals.

Invasive surgeries will be performed under general anaesthesia. Mice will be given pain-relief to manage post-operative pain and kept warm while they recover. Mice will be monitored following surgery and any procedures that may have immediate adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All surgeries will be performed under sterile conditions following best practice guidelines, e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Procedures for establishment and monitoring of tumour growth, metastasis and animal welfare will follow best practice guidelines (e.g. NCRI Guidelines for the welfare and use of animals in cancer research, Workman et al. 2010 Br. J. Cancer). We will also consult the NC3Rs website and Norecopa for information on the 3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly consult the NC3Rs websites, NC3Rs regional programme manager, the literature and colleagues/collaborators for information on the 3Rs. Any relevant advancements that can be implemented will be used.

46. Cell-Matrix interaction 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

Skeletal muscle, Muscle stem cells, Integrins, Extracellular matrix, Muscle regeneration

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 further our understanding of the interplay of cells with their specific extracellular environment, with a focus on skeletal muscle.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Loss of muscle mass during muscle wasting diseases and in ageing is compensated by increased fat and fibrotic tissue. Moreover, muscle loss may lead to diabetes because the most insulin-sensitive tissue in the body is lost. It is estimated that the health service costs just due to age-related muscle loss due to frailty and subsequent falls are £1.7 billion per year. These figures are likely underestimated as they do not take into account secondary

implications of muscle loss such as diabetes and heart disease. Moreover, muscle loss has a devastating effect on the quality of life, especially of the elderly, including reduced motility and eventually loss of independence.

Recent evidence suggests the extracellular environment, or “niche”, of the muscle endogenous stem cell, changes with continued muscle repair or with age and limits its regeneration. This study therefore seeks to determine mechanisms for impaired muscle stem cell functionality in a mouse model in which interaction of the stem cell with its niche is compromised. Although this project is primarily directed at identifying underlying basic mechanisms that are leading to muscle loss, any outcome may be valuable in the future to develop therapeutic strategies to slow down muscle loss.

What outputs do you think you will see at the end of this project?

We expect to generate several publications in well-respected academic journals on how the muscle stem cell is regulated by the extracellular interaction with a long-term understanding of potential methods to maintain muscle mass in ageing and muscle wasting diseases. We in addition plan to communicate our findings to the public, either by press statements or in local science festivals.

Who or what will benefit from these outputs, and how?

We hope to further our understanding of the role the local environment of muscle stem cells plays in development and disease progression and/or prevention. The primary aim of the project is to unravel fundamental basic mechanisms of skeletal muscle regeneration, which have not been studied so far. This information will in the short to medium term mainly benefit researchers in the skeletal muscle field. In the longer term, there is a potential benefit that new drugs and/or new therapeutic strategies based on this work can be developed to combat muscle wasting in disease and in ageing.

How will you look to maximise the outputs of this work?

We collaborate with researchers with expertise in similar areas both locally, nationally and internationally. We will also attend national and international scientific meetings to present our unpublished work, through which we expect to obtain feed-back and advice from the scientific community.

Species and numbers of animals expected to be used

- Mice: 9925

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 exact regulation of muscle development and regeneration can only be achieved in vivo. While we do use in vitro cell culture models, these have major limitations as such

models do not reflect the mechanical forces exerted on the tissue and lack the interactions with other cell types and the environment. Mice are the optimum model as they are similar to human in many aspects and so muscle regeneration can be easily followed in a local muscle of the hindlimb and allow genetic manipulation. For the majority of work adult mice will be used (>2months of age). However, it may be necessary to follow a specific cell fate during late foetal or post-natal development in a small number of mice.

Typically, what will be done to an animal used in your project?

The majority animals (>95%) used in this project will receive injection of substances either via the intraperitoneal route and/or locally administered into a superficial muscle. These are short-term procedures and mice are not expected to exhibit any harmful phenotype.

Insulin and glucose tolerance testing requires that the animals are starved for up to 16hrs, after which insulin or glucose will be injected intraperitoneally. Glucose levels are then measured in the following in a small blood drop obtained from the end of the tail. The mice are not expected to exhibit any harmful phenotype.

What are the expected impacts and/or adverse effects for the animals during your project?

Possible adverse effects that may happen to a small percentage of animals are Some offspring may lose weight due to muscle wasting.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity for this project is moderate, but it is expected that only a small minority of animals (<5%) will experience this severity. The majority of animals (>95%) are expected to make a rapid and unremarkable recovery from the procedures.

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 function and regulation of adult stem cells in muscle tissue under normal and pathological conditions can only be unambiguously analysed in vivo. In vitro cell culture models do not reflect the mechanical forces exerted on the tissue and the interaction with

other cell types and the environment, do not assemble higher order structures such as basement membranes and cannot mimic metabolic changes like glucose or insulin sensitivity and effects on other organs of the body.

Which non-animal alternatives did you consider for use in this project?

Although our work is animal based due to the reasons given above, we are using several in vitro and ex vitro models to answer basic questions

- When possible in vitro culture models will be used for analysis of basic mechanisms (e.g. signalling cascades).
- Immortalized cell lines have been from several transgenic lines to test basic mechanisms (e.g. signalling cascades, effect of glucose).
- Ex vivo models for muscle regeneration are used for underpinning basic concepts of adult stem cell function.

Why were they not suitable?

Cell culture models are a good system to overall reduce animal number as candidates (e.g. signalling cascades) can be pre-tested before they are verified in cell directly isolated from mice. However, although the ex vivo regeneration model will deliver data, how well a muscle regenerates and how the organism reacts to high fat diet can only be achieved in an animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are estimated on our current usage and projected proposed studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use several measures to reduce animal numbers.

- The experiments are designed to use the minimum number of animals necessary. This includes the use of established immortalised cell lines as in vitro culture models for pre-screening studying basic mechanisms and ex vivo methods to analyse basic concepts of adult stem cell function and only bring the most likely candidates into in vivo studies.
- We have calculated the number of animals for each experiment together with a statistician to use the minimum number of mice whilst still receiving meaningful and statistically significant results.

- We will use the Experimental Design Assistant provided by NC3R to design experiments the most efficient way and put stops if necessary.
- When using genetically modified mice, we use homozygous mutant mice for mating wherever possible to minimise unrequired genotypes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- When using genetically modified mice, we use homozygous mutant mice for mating wherever possible to minimise unrequired genotypes.
- We use both male and female mice for our studies.
- We isolate tissue from animals from experimental animals and store it appropriately for later use in histological and immuno-histological analyses and/or share tissue when appropriate with collaborator and colleagues, thereby reducing the need to breed mice specifically for this purpose

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 uses the mouse as model organism, as it is easy to genetically manipulate. The strains required are available without the need to create new lines. There is also a plethora of information about dosing of substances available in the scientific literature, thereby reducing the need for pilot studies to determine appropriate dosages.
- We have experience of all the methods to be used in this application and they have been optimised to cause the least pain and distress to animals and will not cause any lasting harm to the animal other than transient.
- Animals will be humanely killed after the injection of gene deleting/inducing agents and used for in vitro and ex vivo studies of skeletal muscle regeneration.
- A short anaesthesia during which a superficially located muscle will be injected with a myotoxic agent will not cause any lasting harm to the animal other than transient.
- Glucose metabolism on a chow or high fat diet will be tested after starving for a maximum period of 16 hrs by injection of appropriate doses of glucose and insulin, what will not cause any lasting harm to the animal other than transient.

Why can’t you use animals that are less sentient?

Our research requires whole body systems in order to understand the complex physiological interactions of individual and multiple genes on multiple organs of the body.

The mouse is the least sentient animal that is both physiologically and genetically closely similar to human and allows both genetical and pharmaceutical manipulation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- Welfare of the mice will be monitored after the administration of substances on multiple occasions by the experimenters and then inspected daily by qualified animal carers. Aseptic precautions will be taken to help prevent infections.
- Surgical procedures will be carried out according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Analgesic agents will be administered as required after the surgery. In the event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions.
- Altered food composition should cause no adverse effects to the animal. Weight will be controlled weekly and mice will be humanely killed if the weight gain restricts movement to reach food and water.
- Animals will be monitored constantly while on the treadmill or rotarod. Voluntary running of mice on the treadmill will be supported by covering the end of the treadmill with a black bin liner. When testing endurance animals will be removed from the treadmill or rotarod as soon as they avoid running voluntarily.
- Any animal will be humanely killed before signs of distress (e.g. loss of body weight, unkempt fur, abnormal posture, loss of muscle tone) become apparent.
- All procedures applied to animals under this licence are current best protocols and well validated and will not cause any lasting harm to the animal other than transient. We will follow the advice of NC3R in case the protocols will be adjusted to minimise harm.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All our studies follow the guidelines of NC3R which are widely accepted by the scientific community. In addition, we keep up-to-date, by following updates provided by the Laboratory Animal Science Association (LASA) which, together with NC3R constantly publish improved and refined animal procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All researchers involved in this project will be encouraged to subscribe to the NC3R newsletter and website and review current methods as soon as new guidelines will become available. All researchers are also encouraged to subscribe to the Experimental Design Assistant provided by NC3R to design experiments the most efficient way and put stops if necessary.

47. Roles for genomic instability in cancer and aging

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

Cancer, Ageing disease, Genomic instability, Phenotyping, Therapies

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.

Description 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 find genes that regulate genomic instability (GIN). The genes will be researched to find ways to prevent genomic instability beginning and building up.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

- Genome instability (GIN) contributes to many diseases which, if prevented, would lead to disease improvement. Discovering ways to prevent GIN could lead to better treatment of cancer and ageing.
- Our cells are exposed to factors that lead to DNA damage. The environment, such as UV light, radiation and chemicals, can cause the damage. Alternatively, damage can come from within the body during natural processes. For example, when the body uses

water or converts food to energy and other life sustaining components. When these processes go wrong in the body, bad chemicals or molecules can be created that cause DNA damage. The natural processes in the body may go wrong because of faulty genes.

- DNA can be damaged in a minor way but also in a major way. DNA can be damaged at different times when a cell is growing. How the body decides to fix damage to DNA depends on these circumstances and is called the DNA damage response (DDR).
- DDR senses DNA damage and triggers DNA repair, cell cycle arrest or cell death. Failure of these responses results in GIN and the start of disease. This includes developmental disorders such as progeria, an illness where people show signs of ageing and die early. Also, some forms of ataxia, which can affect co-ordination, balance and speech. Such diseases may affect the brain, the nervous system, muscle function and the immune system. They can affect people at different ages, young and old. They may cause degeneration that results in loss of movement, the senses, premature ageing and ultimately death. DDR failure resulting in GIN also leads to cancer and ageing related disease including dementia. A better understanding of how GIN works provides potential routes for disease prevention and treatment.
- There were 9.6 million deaths from cancer worldwide in 2018. Alzheimer's and other dementias led to
- 1.5 million deaths in 2017. As the world population ages the cases of cancer and age-related illness are set to increase. It is estimated that 47 million people live with dementia around the world. Finding suitable treatments for certain age-related disease has proven difficult. We believe that this area of research is crucial for discovering new ways to combat cancer and age-related illness.

What outputs do you think you will see at the end of this project?

Primary outputs:

- New genes of interest will be identified and described. Novel therapeutic strategies will be discovered.
- New mouse strains will be created and mouse strains created elsewhere will be investigated further, providing new information about those strains. New data will be created on the effect of novel therapeutic agents on the process of genomic instability (GIN).
- We will gain new knowledge of how certain genes work. We will also get information of how one or more genes work together. This information will allow us to make decisions on the best way to move forward in researching the many different genes involved with GIN. We will get new information on how certain genes and combinations of genes cause diseases to be worse. Importantly, we will discover gene combinations that will reduce illness and suffering. For example, we will find combinations of genes that will reduce the rate at which a cancer will grow. We will find combinations of genes that will stop or reduce the faulty development and ageing health issues associated with GIN. Biomarkers or gene targets will be identified. These secondary outputs will help us work toward our primary output to find particular drugs to combat the illnesses.

- New cell lines will be derived from the embryos of the mouse strains studied. Newly created mouse strains and materials originating from these mice, including cells and tissues, will be made available to the scientific community.
- Work will be published in peer reviewed journals.

Who or what will benefit from these outputs, and how?

- Mice and tissues produced will be supplied to other researchers. This will include new mouse models and novel genetic crosses with details of their phenotype. This will enhance and speed up the research for these investigators.
- Outputs such as biomarkers could be used in pre-clinical and selected clinical trials. A biomarker is something that can be measured to show what is happening in the body. Measuring biomarkers can indicate the presence of a disease. Cancers can produce biomarkers and so can the body's healthy cells in response to the cancer. Cancer varies between individual patients and using biomarkers can help show what is happening inside the body and identify the best approach for dealing with a particular case. Thus, finding new biomarkers improves our ability to both detect and treat cancer.
- GIN is a hallmark of cancer and ageing and there are millions of people in UK and throughout the world that are affected by GIN related disease. The approach we will employ in this project and the outputs generated will help find new therapies to fight illness in those affected. Our work will provide benefit for those suffering from neuro-developmental disorders such as ataxia. We will be contributing to finding ways to combat dementia, including Alzheimer's and Huntington's. Other specific illnesses targeted include Hutchinson Gilford Progeria and Cockayne syndrome. Ultimately, cancer sufferers and patients of these diseases will benefit along with their carers and society as a whole.

How will you look to maximise the outputs of this work?

All mouse lines we produce will be made freely available. Moreover, we will report all data that will be generated under this licence. Negative data is very informative but seldom published; following quality control and validation, all generated data, positive and negative will be published in open access databases or journals. We will collaborate with other researchers in the field and present findings through meetings and conferences.

Species and numbers of animals expected to be used

- Mice: It is expected that a maximum of 14,000 mice will be used over the course of 5 years, with 9,500 of these being used solely for breeding to generate mice for analysis.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

- Using mice is the best test against data gained in the lab. Using ageing and cancer mouse models is the only approach available for full investigation of the processes involved with DNA damage response (DDR).
- Using the mouse embryo allows us to make the mice we need for our work. The mouse embryo also allows us to do work without the need for live mice. Young mice are used because the genes we study may have an impact on development. Studying young mice is important when looking at illness relating to age because signs of illness can start earlier with age related disease. Much of our work will be done on adult mice since a lot of data can be gained at this stage.

Typically, what will be done to an animal used in your project?

- The mice on this licence will be used to understand the role of genomic instability (GIN) in the promotion or dampening of tumour formation and ageing.
- Some new mouse strains will be created. Different strains of mice will be bred together. Such strains will not have been bred together previously. We will study these new strains to see if they produce mice at the usual rate and whether they develop normally. We will look at the development at different life stages. Individual mice from these new strains may be examined over the first few weeks of their lives, have their weight, shape and size measured. Simple procedures may be carried out on these mice, such as a blood sample or x-ray image. Other mice from the new strains will be used to test behaviour. In these cases mice will undergo non-invasive behaviour tests carried out on several days over a three week period to examine how the brain is working.
- We will breed different strains of mice together to see if one strain stops the signs of disease in another.
- Mice will undergo procedures where tumour development can occur or they may be allowed to age. These mice may carry an altered gene and are monitored to see if they develop a developmental defect, tumour or age related change.
- Sometimes, mice will be injected with cells that produce tumours. All such mice are monitored daily for any signs of developing tumours. The mice will be checked to see if the tumours are making them ill and may be given drugs to reverse the tumour growth. Certain drugs can be given in food, a low harm route. Such mice will be studied for a few weeks.
- Mice will have blood samples taken. Images including x-rays may be taken. Sampling and imaging procedures are quickly carried out as a single event.
- If a mouse becomes sick unexpectedly it will be humanely killed and the mouse examined to see why it was displaying these symptoms. Samples will be collected for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

- Many mice will suffer no adverse effect during the project.
- Some mice will experience no more than a transient feeling of pain or suffering.

- Few mice will develop at a slower rate and/or have developmental abnormalities, for example curvature of the spine. These effects may develop gradually over weeks and will not be allowed to prevent the mouse from feeding and drinking.
- Some mice will form tumours. Tumours may last for a few weeks but will not be allowed to stop mice carrying on with normal behaviour. For short periods, the body weight of the mouse may increase or decrease in the presence of a tumour. Mice may move differently or have uncoordinated movement. Breathing may be more difficult. Skin could become more irritated in the presence of a tumour.
- Some mice may show signs of ageing earlier. There may be gradual weight loss, over several days, and loss of fat and muscle mass.
- Certain mice may experience loss of coordination or seizures. This is expected to be rare and for short periods.

Expected severity categories and the 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 large proportion of the mice, possibly more than 5000 mice and particularly on the breeding licence, are not expected to suffer any adverse effect (sub-threshold).
- About 30% of the mice we propose to use will experience pain or suffering that is classified as 'mild'. Particular mouse strains known to develop adverse effects or an illness because of their genetic alteration will not be allowed to develop more than short-term mild pain, suffering or distress. The genetic alteration in the mouse may result in a difference in growth rate, size or behaviour. However, these mice will have a normal lifespan and will be able to freely move and feed. These mice will have only one or a limited number of procedures carried out on them, which will result in no lasting adverse effect. Where these mice are used for more than one test, it will be ensured that the combined affect causes no more than slight or transitory pain or suffering.
- About 35% will be classified as 'moderate' severity. These mice may shows signs of disease but will not become severely ill or die as a result. Mice may lose more of their body weight, up to 15% weight loss. Some mice may have a more significant loss of normal movement or there may be more persistent evidence of an illness. Some mice will develop tumours. Growths will be monitored to ensure they do not go over a certain size and do not make the mouse severely ill or significantly impair movement.
- Surgical procedures, for establishing new mouse colonies, will result in pain for no more than a few hours and include administration of pain relief.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

- We can test in the laboratory without using animals to provide useful data on how diseases work in humans or animals. We can also run tests without using animals to see how different genes work together to make a disease better or worse.

- Particular cells from animals can be used to test the effect of drugs that could stop a disease. With cancer and ageing as studied in this project, we can only confirm that lab tests have worked well by testing further with mice. When mice are used to find genes that affect cancer or ageing or to prove a drug works against a disease, it provides an avenue for finding medicines for humans.

Which non-animal alternatives did you consider for use in this project?

- Extensive analysis without using mice is integral to the project and is always the first option considered when new biological areas of interest are identified. Organoids from both human and animal origin are our first alternative to using mice.

- Organoids are 3D organ-buds that can be grown in the lab. They can be made from the tumour and normal tissue of a patients cell. Organoids enable us to study how tumours develop and how they respond to treatments. Tests using organoids can reduce the number of mice needed for experiments and improve the chances of successful experiments using mice.

Why were they not suitable?

Accumulation of GIN is a multi-step, highly complex process involving cell and tissue interactions. GIN accumulation can act together with multiple genetic abnormalities. GIN associated disease including developmental disease, cancer and ageing affects all organ systems in a highly interconnected fashion. This means that GIN and the associated diseases cannot be fully modelled without a whole organism such as the mouse.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 previous experimental work for similar programmes of work done on licences held in other establishments whereby 15,000 mice were created and used over an 8 year period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Mouse experiments are not carried out until there is sufficient evidence from testing in the lab. Animal sample size calculations are made to ensure as few mice as possible are used whilst still achieving good results. Online resources from trusted and experienced organisations provide software and templates that help with this.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Where possible we shall import existing mouse lines rather than generating new ones. We will utilise the archive at our previous establishment, where our current colonies are cryopreserved, and will use wildtype mice that are surplus wherever available. In general, we will order lines in as frozen sperm or embryos for recovery. This will reduce the number of mice used for breeding between facilities as well as remove the harms related to the stress mice are under when in transit.

- For certain studies, such as when cells are injected into mice for cancer experiments, we will use commercially available mice. This will reduce the need to produce and breed mice at the facility.
- All mouse lines will be archived so that they may be distributed to other researchers worldwide. This will reduce the number of animals used globally, as fewer animals will be required to re-generate these archived lines.
- In some circumstances, such as when certain mouse lines have been only recently created, less published data will be available thus we propose to perform small pilot experiments to determine the final experimental design.
- Wherever possible, multiple experiments will be performed on the tissues collected from an individual mouse so as to maximise the use of the mouse.
- All data generated from our research on the mice will be published in scientific journals available to the whole scientific community, reducing duplication of production resources and phenotyping procedures elsewhere. Wherever possible, the results of experiments that involve large datasets will be made publically available to serve as a resource for other scientists and clinicians.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 cancer and ageing will be used during this project.

- Mice will only be bred if they are required for experimental analysis, distribution or archiving. Furthermore, the methods selected to analyse mice are all standard, robust, well validated tests that are commonly used in high throughput projects.

Why can't you use animals that are less sentient?

Mice and humans have the same basic organ systems, skeleton and reproductive cycles. These similarities, coupled with the technology available to manipulate the mouse genome, make the mouse the best to copy human diseases. There are no, less sentient animal models for tumour growth that can be used. Using non-mammalian species of lower neurophysiological sensitivity is not possible since they lack appropriate tissue physiology. Using mice at an immature life stage would not be possible as we need animals to be mature since we are often looking in to the effect of ageing.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- Whenever guidance materials are updated with recommended refinements, we will strive to include them into our procedures. We will regularly review literature and discuss possible refinements with peers and experts.
- At all times, mice are handled calmly and following advice for best practice, for example tunnel handling.
- Where mice are imported in to the facility, they will always be given a minimum of one week for acclimatisation prior to use in procedures or further movement.
- Non-surgical methods of transferring embryos will be considered for mouse production. This will be assessed based on the ability to recover suitable numbers of live mice. All surgery will be undertaken in full compliance with Laboratory Animal Science Association aseptic technique guidance to minimise infection risk. Mice will always be provided with pain relief for surgical procedures, prior to commencement of surgery and, if required, following surgery. The group has over 10 years experience in refining surgical procedures, including the development of improved aseptic technique and rigorous analysis of surgery competency. We will continue to refine skills and techniques to improve surgical outcomes.
- When creating new lines we will use optimal methods for design and production. For example, it is our intention to create new GA mice using CRISPR technology, which significantly reduces the number of animals required for both surgery procedures and later during breeding.
- Mice which have had anaesthesia will have frequent monitoring until fully recovered and additional post recovery checks in animal holding rooms. When general anaesthetics are necessary, practicable combinations with the least adverse effects will be used. For example, gas anaesthetics will be used wherever possible.
- It is our intention to incorporate image recording technology for monitoring of some behaviour tests to improve our ability to successfully blind the experiments.

- Tumour growth experiments are carried out with extra monitoring of the mice involved. Mice will be checked using advanced welfare checks. These checks will be done more regularly than standard welfare checks and by staff trained to spot particular problems. Monitoring protocols can be changed or updated based on the particular mouse strains being looked at. For example, mice expected to experience adverse effects at an earlier age will have monitoring more regularly at an earlier stage of their life. Similarly, mice expected to develop tumours quickly will be monitored more regularly and at an earlier stage of the study.
- When dosing mice for treatment drugs via oral gavage, we will be using a refined dosing technique using plastic/silicone gavage tubes that reduce trauma and make visualising delivery easier.
- If there is a requirement to dose mice with treatment drugs beyond 30 days, usually administered via the intraperitoneal route, we will consider the option of mini-pumps for continuous dosing as an alternative.
- We have experiments where we are able to administer drugs via the mouse diet. Choosing this dose route is a refinement compared with other routes, such as in water or injection, where harms are known to be greater.
- Prior to recovering strains for breeding we will examine health observations previously recorded on a database that will allow us to accurately predict the onset of certain clinical signs of illness. We will then make notes on the database used by animal care staff so that they can quickly respond to welfare concerns. Furthermore, we will create a simple reference document listing all strains that will be used on this licence, including description and time of onset for particular health concerns.
- For all tests it is important that the animal has no additional stress, therefore mice are handled calmly and habituated to testing rooms as well as arenas if possible.
- Where mice are housed in modified cages for testing it will be for the minimum time needed to gather data. Mice will be removed from test scenarios if suffering from an adverse stress reaction, or other unexpected adverse effects.
- Technical refinements will be developed throughout the project and disseminated to other researchers and collaborating institutes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

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 (2017).

We will be following ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I, and delegates from the research team, will work together to adopt new developments in the 3Rs at the establishments the work will be carried out in. We will work closely with named persons and managers involved with promoting welfare, where possible, attending

events promoting the 3Rs hosted locally and through institutions such as NC3Rs, LASA and the IAT. The International Society for Transgenic Technologies (ISTT) provides additional contacts and materials for keeping up to date with production technologies emphasising the 3Rs.

Any new methodology, techniques or practical refinements will be tested against established techniques and where results are not compromised and animal welfare improved will be implemented accordingly.

48. The role of scavenger receptors in the development and progression of hepatocellular cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Liver cancer, Immunity, Scavenger receptors

Animal types	Life stages
Mice	juvenile, adult, pregnant, aged, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the role of scavenger receptors in the development and progression of primary liver tumours (also known as hepatocellular cancer) and whether they have an impact on the ability of the immune system to control cancer. To test whether the administration of therapies to block scavenger receptors can stop tumour growth in the context of liver disease and their potential as an immunotherapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 commonest form of primary liver cancer is known as Hepatocellular Cancer (HCC). HCC is rising dramatically around the world and it is now the second leading cause of cancer related deaths globally. Most patients present with incurable disease and have limited therapeutic options. The majority of patients with hepatocellular cancer (90%) have an underlying liver disease which leads to liver scarring. It is now clear that the cancer is protected from the immune system within the scarred liver.

We need better understanding of how HCC is protected from the immune system. If we can reverse the process, we can then activate the immune system to kill the cancer. Recent trials with immune therapies (checkpoint inhibitors) have proven that this approach could work, but are only effective in less than 20% of patients.

What outputs do you think you will see at the end of this project?

Hepatocellular cancer (HCC) is now classed as the second leading cause of cancer related deaths globally. The incidence is rising and the majority of patients present with advanced cancer and current therapies for these patients only prolong survival for a few months. My programme of work sets out to understand how the immune environment around liver cancer is regulated by scavenger receptors. The use of immunotherapy has shown promising results in early phase clinical trials but less than 20% of patients respond to this therapy. Our work will add significant knowledge to understanding how HCC evades the immune system and will be important for the scientific, medical and pharmaceutical industry. Specific outputs at the end of this work will be publications on how scavenger receptors play a role in the development and growth of hepatocellular cancer. The proposed work will identify mechanisms of cancer progression as well as new therapeutic targets and intellectual property (IP).

Who or what will benefit from these outputs, and how?

In the short term, the data will likely be used by basic scientists and clinician scientists to inform the design and outputs of their own experiments.

In the medium term The scientific community will benefit from these experiments because they should provide better understanding of the pathways that regulate cancer development in the context of inflammation and fibrosis, a major worldwide problem which is still poorly understood.

In the long term we also hope to identify scavenger receptors as new therapeutic targets for treating hepatocellular cancer. Blocking the action of these receptors may be effective in stopping and reversing tumour growth and may also improve the efficacy of current standard of care treatments. To facilitate this, we are skilled and have a strong track record of working with technologies based around the use of human material to interrogate

disease mechanisms, and our proposed animal investigations form an essential partnership to these studies to inform our understanding of cancer development in the setting of inflammation in several organs. We will have the potential of moving rapidly into early phase clinical trials, thus benefits of our work include transfer of knowledge, as well as improvements in treatment for UK patients and the healthcare industry.

How will you look to maximise the outputs of this work?

The data generated by these studies will be published in high impact journals conforming with ARRIVE guidelines provided by the NC3Rs, and presented at national and international meetings and conferences. The development of large datasets will be made available in open access data repositories for external investigators. Findings from this project will be shared with worldwide researchers through communication, including interdisciplinary collaborations, seminar engagements, and conferences. Regular local meetings provide a forum to communicate results to immediate colleagues including clinicians and members of the public attending local hospitals providing additional routes to communication at the local level. Communication of significant research findings will be enhanced with support from dedicated press teams. We also have international collaborations, the aims of these collaborations are to enable progress in translational cancer research through the development of tools, platforms and skills that form the foundations of such research.

These collaborations are therefore an ideal platform to maximise the output from this work.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

I have chosen this species as previous reports have demonstrated that murine models of liver cancer develop tumours which are pathologically similar to human disease. Several studies have shown that factors that drive the development of human liver cancer, especially underlying liver disease, can be reproduced in the mouse. This is vitally important to ensure the experimental findings have the

potential for translation to humans. The mice will be started on the experiments at an early stage so that the tumour development reflects the situation in humans, where tumours develop gradually on the background of liver disease, which occurs in 90% of patients with this type of tumour.

Typically, what will be done to an animal used in your project?

Some animals may receive injections or a gavage to induce genetic mutations. All animals will undergo a single injection into the abdomen at the start of the experiment with the majority then being started on a special diet. In combination, this will lead to mice developing liver tumours on a background of liver inflammation which reflects the development of liver cancer in humans. Animals will be kept for up to 12 months. During that time they may undergo blood sampling and receive further injections of potential treatments.

What are the expected impacts and/or adverse effects for the animals during your project?

Fundamentally, the animals will develop liver tumours, but these are not expected to cause any adverse effects over the planned experimental duration. Animals will however be closely monitored for adverse effects and if seen, animals will be humanely killed.

Where animals are receiving an altered diet, this should not cause any significant adverse effects; over a 12 months period the mice gain weight but it will not impinge on normal behaviour. Animals may develop a greasy coat leading to skin irritation, but steps are taken to minimise this. If it cannot be alleviated then animals will be humanely killed.

Most compounds to be used in this project have been successfully used in mice previously with no adverse effects, however we cannot rule out adverse effects from novel compounds being investigated as potential therapies. In this case, animals will be closely monitored for signs such as reduced mobility, shivering, hunched posture, piloerection, and failure to eat or drink. If such signs are detected the animal will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- The expected severity for all animals under this project licence is moderate. This is because the experimental diet will cause weight gain and gradually cause liver inflammation but will not be allowed to progress to liver failure. The liver tumours that develop during the 12 month period will not be allowed to grow large enough to cause significant discomfort or distress in the animals.
- Even in those animals where potential therapies are shown to be successful, they will have still received a combination of procedures that meet the moderate severity category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of an animal model is vital in order to mirror the multicellular environment of human cancer and its interaction with the whole immune system. We have studied scavenger receptors in human tissue but to demonstrate that they affect the growth of cancer and to confirm its mode of action requires animal models.

Which non-animal alternatives did you consider for use in this project?

Non-animal studies with human tumour cell lines and primary human immune cells were considered as an alternative.

Why were they not suitable?

Human liver cancer cell lines are available but the major criticisms with these experiments are that they do not take into account the multicellular environment and surrounding liver inflammation that is characteristic of human disease. Non-animal alternatives have however been used to inform project direction and replaced aspects of the work which otherwise would have required more animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used previous data from both our laboratory and that of our collaborators, plus published information, in order to inform our estimations. We also use pilot studies to help inform project design and the number of animals required to obtain statistically robust data.

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 published literature to estimate the number of tumours per mice using this model of liver cancer and utilised statistical tools for power calculations to ensure there are adequate numbers of

animal in each group to answer the research question. These calculations have followed guidelines outlined by the NC3Rs.

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 taken expert advice from our animal facility regarding the breeding of genetically altered lines and the minimal number required to maintain a colony. We will optimise the amount of information from each animal by studying changes in tumour growth,

metabolism and immunology. We also plan to run pilot studies to ensure the optimal duration and time points for treatments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Induction of liver tumours: This involves the injection of a carcinogen, diethylnitrosamine (DEN). DEN can be injected at various ages to induce liver cancer- we have chosen a time point that requires only a single injection as opposed to repeated procedures. The scientific endpoint is such that the mice should not develop significant adverse events, as the tumours do not grow to a size that causes distress. However, mice will be monitored throughout the study using a clinical score sheet which will include – weight, body condition, general health e.g. coat condition, palpation for evidence of liver tumours .

High fat choline deficient diet. There is a specific deficiency of choline incorporated into the diet which contributes to fatty liver inflammation and low grade scarring of the liver. We do not anticipate any significant adverse effects as the mice will not develop features of liver failure. This approach was selected as it achieves the required level of inflammation via the diet, rather than by administering multiple injections, unlike other methods.

Why can’t you use animals that are less sentient?

This project aims to understand how a family of molecules called scavenger receptors control the immune system in the setting of cancer. Mice have been chosen for these studies as the main components of their immune system are also found in humans; this is essential as the focus of the project is to understand how the immune system influences tumour development. Tumour development in less sentient animals does not incorporate the complex cellular cross talk between the tumour and the immune system that can be modelled in mice.

How will you refine the procedures you’re using to minimise the welfare costs (harms) for the animals?

We will closely monitor animal body weight, behaviours, and general condition to ensure that the animals do not undergo any undue suffering. This includes the use of welfare score sheets tailored to these models. Where animals may develop a greasy coat and skin irritation due to diet, we provide the diet on the floor to prevent it dropping onto their backs, and provide extra bedding. Where possible, animals are handled using the refined handling techniques promoted by the NC3Rs.

Where there is a choice of route of administration, we will always use the most refined whilst scientifically justified.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use LASA dosing guidelines and blood sampling recommendations.

Any publications resulting from our research will be reported in accordance to the ARRIVE2.0 guidelines and we will use the PREPARE guidelines when planning our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I have already attended workshops by the NC3Rs and will continue to stay engaged with the NC3Rs through their website. We are also part of an international collaboration for liver cancer. One of the main aims of this collaboration are to develop new models of liver cancer to reduce animal experiments using laboratory methods with human tissue including organoid cultures and whole tissue specimens. I attend regular meetings and training workshops organised by this consortium, providing an opportunity to implement these techniques in my research. I will also be attending national and international scientific meetings to remain up to date in the latest non-animal techniques used to study liver cancer.

49. Exploring the role and function of fibroblast subsets in inflammatory arthritis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Arthritis, Therapy, Fibroblasts, Inflammation, Refractory

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the role of fibroblast subsets in the pathogenesis of inflammatory 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?

Current treatments for inflammatory arthritis target immune cells or the substances they produce. Whilst this approach is effective, some patients do not respond adequately to current treatments and/or relapse following the withdrawal of therapy. As a result, there is a need to develop new therapies that target different disease pathways and particularly those driving disease persistence. We have examined the role of fibroblasts, a key cell type present in the lining of the joint and demonstrated the cellular heterogeneity of these cells in the joint. During inflammation, some of these fibroblast subtypes produce signals that attract immune cells to the joint resulting in persistent inflammation. These immune effector fibroblasts are highly enriched in the joints of patients with rheumatoid arthritis (RA) and therefore represent an attractive therapeutic target.

There are many questions that remain before we can translate these findings into clinical studies for patient benefit. Firstly, how do the types of fibroblast within the joint affect the outcome of inflammation and damage? How do the proportion and type of fibroblasts change over time and in response to treatment? What is the exact function of the individual fibroblast subtypes and what mechanisms regulate their phenotype? This proposal outlines a series of experiments to determine the type of fibroblasts associated with poor treatment response, the function of these fibroblasts and the mechanisms that regulate their behaviour and phenotype. The findings from this research will provide a clear rationale for the therapeutic targeting of specific subsets of fibroblasts in human disease.

In particular, our aim is to identify which subsets of fibroblasts are most important in the development of inflammatory arthritis and whether specific subsets of fibroblasts determine the response to therapy and underlie the development of treatment refractory RA. We aim to explore whether modulating fibroblast function improves disease and ultimately which of these specific fibroblast subsets to target therapeutically.

What outputs do you think you will see at the end of this project?

Project outputs will include:

- advances in scientific knowledge on the role of immune effector fibroblasts in treatment refractory rheumatoid arthritis.
- scientific and lay publications of our findings and methodology.
- presentation of our data and/or methodology to the wider scientific community through conference oral and poster presentations.
- identification of novel targets or agents that alter the behaviour and phenotype of fibroblasts in the joint, which can be taken forward to develop new therapies.

Who or what will benefit from these outputs, and how?

Short-medium term

Scientific developments and innovations leading to our enhanced understanding of the role of immune effector fibroblasts in inflammatory arthritis described in this licence will have an immediate impact on the ongoing projects at the host organisation, and also further afield upon dissemination. Moreover, the project aims to improve our understanding

of the processes driving pathology in chronic disease, specifically disease pathways mediating the persistence of joint inflammation that we anticipate will underpin the development of the next generation of anti-rheumatic therapies.

Medium-long term

We intend to invite external seminar speakers who have an interest in the role of fibroblasts in chronic inflammatory diseases to our organisation, with the view of fostering further collaborations based on the concepts and ideas incorporated in this proposal. We envisage that these collaborations will occur during and following the completion of this project, and therefore represent a medium to long-term impact of this work. Using these collaborations, we will develop the infrastructure needed to translate these findings into clinical trials and ultimately, clinical practice, therefore fully exploiting the translational potential of this work.

Long-term

Clinical academics, pharmaceutical companies and patients directly will benefit from advances in our understanding of disease pathology and particularly why some individuals with arthritis do not respond to currently available treatments, along with identification of novel targets, which can be taken forward to develop new therapies. We will develop collaborative networks to realise the translational potential of our findings over the subsequent 5-10 years following the completion of this project.

How will you look to maximise the outputs of this work? Dissemination of information

We will work with the relevant teams within our Establishment to facilitate communications and resulting impact. We plan to use several routes to disseminate our findings to the wider scientific community, industry and the public that will facilitate end-user engagement, namely:

Peer-reviewed publication: we aim to publish high impact papers based on the findings generated from this project licence. In addition, our group has a strong tradition of publishing methodology papers; and negative data to ensure that groups do not unnecessarily repeat experiments that either technically are flawed or biologically yield the null hypothesis.

Presentations: we and our collaborators will present data at internal seminars along with national and international conferences.

Dissemination via international societies: we and our collaborators are active members of various scientific societies, allowing our findings to be disseminated to the wider scientific community in societal magazines and training workshops.

Enhancement of public understanding and engagement with research

We will take advantage of several public engagement events organised by the Establishment and local charities to facilitate the public’s awareness of our research:

Science “pop-up” activities: we actively participate in “Meet the Scientist” tours and host stalls at local Science Festivals.

Lay Resources: we would develop lay resources, in collaboration with our patient/public research partners (PRP), for publication. Additionally, we will continue to involve patients and the public in the delivery and dissemination of research generated from this project.

I actively engage with our rheumatology patient research partners in all stages of our research including research strategy and planning, as well as dissemination to lay audiences. These partnerships are pivotal in developing our ongoing research strategy and ensuring our work is disseminated to lay audiences.

Clinical networks and translational collaborations

My team and I are active members of several multi-institute research centers and will be able to present our findings at least twice annually at ongoing Centre seminars. We will also attend clinical conferences in our field, where we will present our data and foster collaborative opportunities for translational research across the fields of rheumatology, ageing and chronic inflammatory diseases. As a team of both clinicians and scientists we have access to unique patient cohorts and therefore the expertise and ability to translate findings rapidly to early clinical studies.

Species and numbers of animals expected to be used

- Mice: Mouse ~6,000 over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

Mice are the best model for the study of persistent inflammatory disease because:

The main components of their immune system are shared by humans; this is essential where immune responses as opposed to the function of individual genes is being studied and thus, will produce satisfactory results.
An extensive range of reagents are available for analysis of immune responses in mice and including genetically altered mice.

They are the most acceptable animal models that show the least degree of neurophysiological sensitivity and will suffer the least pain, suffering, distress, or lasting harm.

There are no other alternatives to this work. We are employing models that have been refined and streamlined as much as possible by our collaborators at our Establishment and beyond.

Typically, what will be done to an animal used in your project?

For arthritis models - the majority of animals (80%) will undergo general anaesthesia (up to 10 minutes), daily handling for scoring and calliper measurements of joints and limbs; administration of an agent or cells to determine cellular function or therapeutic efficacy and will be killed up to week 8 via schedule 1 method or non-schedule 1 for withdrawal of fluids (e.g. blood).

Each experimental model will be monitored daily following intervention and mice will be assessed for any signs of distress with supportive measures provided. Procedures will be undertaken using the most appropriate anaesthetic and analgesia will be given. The mode of substance administration will be chosen to cause the least harm and distress to the mouse. Any new substances or route of administration will be tested in a small pilot study and the mice monitored daily for signs of distress.

Humane endpoints will be strictly adhered to at all times.

What are the expected impacts and/or adverse effects for the animals during your project?

The maximum severity limit in this licence is moderate. Possible adverse effects include irritation from local injection, pain from joint inflammation, weight loss or mobility problems. Any animal showing deviation from normal behaviour as judged by; body weight, body condition, general and coat appearance, gait or behaviour will be treated with pain relief and we will administer food supplements such as gel and mash. Before pain and inflammation exceed a moderate severity level, animals will be killed to prevent any on-going pain or suffering. All animals will be killed at the end of the protocol with the exception of protocols 1 and 2 (Breeding and maintenance of genetically modified 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)?

This project will run over 5 years and will use mice as the experimental animal. In this project, the majority of the mice used will be genetically modified with a non-harmful phenotype (protocol 1). These mice will be used to maintain breeding colonies or will be culled by a schedule 1 method for tissue collection. Over the duration of the project we expect ~30% of the mice will be used for this part of the project. A further ~30% of mice will be bred under protocol 2 - genetically modified with a harmful phenotype. The majority of these animals will be used to maintain the colony of which half will carry the harmful transgene. Over the 5-year period we expect that approximately ~10% mice will be used for cellular transfer experiments (moderate), ~10% for monoarthritis (moderate) and approximately ~20% for polyarthritis models (moderate).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to study the inflammatory response, three components need to be examined: time, place and cell type. While place (organ) and cell type (leucocyte or fibroblast) can be examined relatively easily in humans, it is difficult and, in some cases, unethical to perform multiple biopsies, and adoptive transfer experiments in humans with arthritis without underpinning preclinical data that support therapeutic utility. Furthermore, as manipulating fibroblast biology has the potential to affect systemic immune function, we will need to determine whether manipulating fibroblasts as a therapeutic target affects the innate and acquired immune response and this requires testing in different mouse models and those that most closely resemble aspects of human disease. This step is essential in order to ensure that the translation of our findings to humans passes through the appropriate clinical regulatory stages before use in patients with arthritis.

Which non-animal alternatives did you consider for use in this project?

Over the last decade we and our collaborators have pioneered a range of in vitro co-culture models that have furthered our understanding of leucocyte-fibroblast cell interactions in chronic inflammation.

However, we have now reached a point where we cannot proceed to test our ideas without resorting to animal models of arthritis and bone damage as our in vitro findings must be validated in licencing authority approved animal models before they enter clinical trials and are tested on patients. We also need to provide a clear rationale for the therapeutic targeting of these cell types in inflammatory arthritis and that requires a detailed understanding of their role in different aspects of disease.

Why were they not suitable?

Our in vitro models are unable to fully recapitulate the joint architecture in its entirety and how it is altered during disease and especially over time in different phases of disease. Moreover, our current findings must be validated in licensing authority approved animal models before they enter clinical trials and are tested on patients. In some cases, this work represents preclinical therapeutic (drug and/or cell) efficacy studies required prior to embarking on human clinical trials. There are no other in vitro or in vivo alternatives to this work. We will continue to collect as much in vitro evidence as possible before embarking on animal experiments, using it to inform and refine experimental design.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These 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 specific mathematical calculations based upon previous studies and the likelihood of our interventions producing positive results, to estimate the number of animals we will use in our study. For all experimentation, the lowest possible number of animals will be tested whilst ensuring that the experimental result is robust.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A key strength of our work is that it combines both human and animal models so that each can be used to inform the other and therefore minimize an over reliance on mouse models of disease. This will give us the option to stop the line of research at any stage where our findings fail to show any significant increase in our understanding of chronic synovial inflammation. We are running these studies in parallel with studies that explore the same cell populations in immune mediated inflammatory diseases in humans.

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 put in place careful breeding procedures and also, strategies to reduce the clinical symptoms of arthritis and inflammation in such mice by treating them with anti-TNF antibodies in the same way that therapy is given to humans with rheumatoid arthritis. By doing this, we will reduce unnecessary suffering for the mice and therefore, the number of mice needed in our breeding programme as litter mates can be bred for longer periods of time. Where possible, tissue will be archived for future use.

New interventions will first be tested for efficacy using in vitro models prior to use in vivo. Where new routes of administration or new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments. Subsequently these pilot data will be used in the specific mathematical calculations described above to ensure that we use the minimum number of animals needed to obtain statistically significant results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 best model for the study of persistent disease because:

The main components of their immune system are shared by humans; this is essential where immune responses as opposed to the function of individual genes is being studied and thus will produce satisfactory results.

A wide range of wild type and genetically manipulated strains of defined genetic makeup are available.

An extensive range of reagents are available for analysis of immune responses.

They are the most acceptable animal models that show the least degree of neurophysiological sensitivity and will suffer the least pain, suffering, distress or lasting harm.

There are no other alternatives to this work.

We have refined and streamlined as much as possible the models of arthritis that we use. Importantly we will use a research strategy that includes the use of different models of inflammatory arthritis to more accurately recapitulate a specific phase or aspect of joint disease, depending on the scientific questions of that particular experiment. This targeted approach will limit the use of persistent models of joint inflammation to only those experiments where it is needed to model that specific aspect of the disease.

Where necessary, males carrying the mutation will be used for breeding with WT females as this means the female does not have the burden of the effects of the mutation alongside pregnancy.

The maximum time a single mouse is likely to have arthritis is 5 weeks as the arthritis is self-limiting. We will give mice treatments that lessen pain and discomfort that might occur as part of the inflammatory response. This will include pre-emptive treatment with opiate analgesia. We have also adapted treatments used in patients with arthritis (e.g anti TNF biologic therapies) for use in mouse strains that have a genetic predisposition to develop arthritis. We have put in place mitigations as described below in the 3Rs to ensure that any suffering by an individual animal is minimized.

Why can’t you use animals that are less sentient?

Less sentient animals do not possess the same sort of skeletal structure that composes the joints, and often their vascular tree and immune system do not fully represent that of humans. Small rodents are the lowest mammals that can be used to recapitulate the human immune systems response to joint inflammation.

How will you refine the procedures you’re using to minimise the welfare costs (harms) for the animals?

We have refined a number of our protocols over the last five years.

We will use established reagents and protocols that we have developed and refined over the last 5 years to treat the mice. Therefore, we will not need to perform unnecessary toxicity studies and will be able to use the lowest doses of agents that are well tolerated.

We have refined and streamlined as much as possible the models of arthritis that we use. Importantly we have very clearly defined humane endpoints. We have refined procedures of cellular transfer, including by intra-articular injection so the smallest volumes can be injected.

Where necessary, male mice, transgenic for the gene of interest will be mated with wild type females in order to exclude indirect effects on the progeny derived from gene overexpression in the pregnant female.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local AWERB. Following every experiment and regularly during group meetings we will review our procedures from welfare standpoint to identify any potential for refinement.

We will also follow the published literature on arthritic models - for example the case for using different arthritis models to model the different aspects of inflammatory pathogenesis is extremely well made and described in Vincent et al. 2012. Moreover, refinement of the injections for the induction of collagen induced arthritis has been described in Hawkins et al. (2015) and we have implemented these in our work to date and will continue to do so in this project.

If undertaking a systematic review, we will use SyRF, the free online platform for researchers, to perform a systematic review and meta-analysis of animal studies. This will allow us to keep up to date with any improvements in protocols and techniques which may reduce or replace the use of animals.

Finally, we will follow the LASA guidelines Guiding Principles for Preparing for and Undertaking Aseptic Surgery (www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf) and by the Home Office Minimum Standards for Aseptic Surgery (www.procedureswithcare.org.uk/ASMS2012.pdf) when undertaking aseptic surgery and providing analgesia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I or a member of my research team will attend local 3Rs events and sign up to the NC3Rs newsletter. We will also be reviewing the literature on regular basis in our journal clubs and through this network we will discuss any refinements that could be applied to our own work.

50. Understanding the role of androgens in raised intracranial pressure in vivo

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

Androgens, Intracranial pressure microchip, Idiopathic intracranial hypertension, High-fat diet, Cerebrospinal fluid

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?

Test the effects of androgens (dihydrotestosterone) in the presence and absence of obesity on intracranial pressure (ICP) in a mouse model using minimally invasive intracranial pressure microchip monitoring.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue **after the project has finished.**

Why is it important to undertake this work?

Idiopathic intracranial hypertension (IIH) is characterised by raised intracranial pressure and causes headache and a considerable risk of blindness. It predominantly occurs in obese young women with disease burden rapidly rising in accordance with the epidemic of obesity, driving escalating healthcare costs (£450 Million/ year by 2030). Despite the incidence increasing, physiological mechanisms of idiopathic intracranial hypertension are unknown and therefore targeted treatments have not been established.

Our previous work demonstrated that idiopathic intracranial hypertension patients have a unique signature of androgen excess and provided evidence that androgens can modulate cerebrospinal fluid secretion via the choroid plexus. This work was carried out in vitro on rat choroid plexus cell lines and human serum and cerebrospinal fluid samples. These findings implicate androgen excess as a potential causal driver of raised intracranial pressure in idiopathic intracranial hypertension.

What outputs do you think you will see at the end of this project?

This basic research project will lead to better understanding of how androgens impact intracranial pressure regulation. Furthermore we will evaluate how androgens and obesity may synergistically impact intracranial pressure.

Knowledge gained from this project will have direct translational relevance to idiopathic intracranial hypertension patients by demonstrating whether androgens do or do not alter intracranial pressure. This will potentially lead to future therapeutics targeting androgen metabolism in idiopathic intracranial hypertension. Currently there are no targeted therapies for idiopathic intracranial hypertension. The current drugs available are off-licence and merely alleviate disease symptoms but do not address the underlying disease pathogenesis. There is consequently an unmet need to develop targeted therapies in idiopathic intracranial hypertension to help reduce the risk of blindness in this disabling disease.

Outputs of this information will be through sharing our findings at national and international conferences, through collaborations with other researchers, and by publishing in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

The work will add to the knowledge of androgen effects on idiopathic intracranial hypertension. In the short term the outputs will accelerate scientific progress towards a greater understanding of the cause

of idiopathic intracranial hypertension as well as potential therapeutic approaches for the disease in the future.

In the medium term the impact of this project will open new avenues for developing therapeutic targets for idiopathic intracranial hypertension. Our evidence may thus accelerate trials and drug development. Ultimately, in the long term, the goal is to bring efficacious therapies into the clinical environment for idiopathic intracranial hypertension patients.

How will you look to maximise the outputs of this work?

To maximise the outputs we are collaborating with different groups internal and external to this establishment that will lend their expertise and knowledge to our work. We will publish our work in peer-reviewed journals and present at conferences to disseminate new knowledge including reporting of unsuccessful approaches. We will also share best practice of new techniques and protocols we have developed and validated to

collaborators. We will engage with the public through events organised by the establishment to showcase our research.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

We are using a mouse strain that has been previously used for androgen excess experiments as well as high-fat diet experiments and consequently we have an established expertise with this type of animal. We have chosen young adult mice as they will have grown sufficiently to allow implantation of an osmotic minipump and intracranial pressure microchip. Furthermore this mimics the human condition where disease onset is typically in young adulthood.

We will choose mice at a consistent age to ensure standardisation between experimental arms.

Typically, what will be done to an animal used in your project?

Intracranial pressure microchip monitoring has been established with our collaborators. We have noted that intracranial pressure can be monitored continuously whilst also continuously assessing animal movement thereby enabling analysis of intracranial pressure to be quantified both during activity and inactivity. In order to attach the microchip a small hole (0.5mm) is drilled through the skull (without piercing the dura) above the ventricle of the mouse brain. The intracranial pressure microchip is sealed in place using Epoxy resin (nothing penetrates the burr hole or goes into the mouse’s brain, the burr

hole acts as a fluid filled reservoir over which pressure can be measured). Mice will be allowed to recover for a minimum of 7 days following surgery.

To examine the impact of androgen excess on intracranial pressure, young adult mice will be implanted under the skin with a slow release device which will deliver DHT or placebo for up to 6 weeks. To enable monitoring of intracranial pressure the same mice will also have an intracranial pressure microchip implanted a minimum of 7 days prior to androgen device implantation.

Intracranial pressure will then be monitored in freely moving mice twice a week for three hours by attaching the chip to a wire attached to a computer, this will allow the mice to behave normally during the recording period. To confirm the androgen phenotype longitudinal monitoring will be conducted over the 6 weeks including oestrus cycle monitoring until cycle arrest is confirmed, glucose tolerance testing for diabetic function and blood collection for steroid hormone levels. For the glucose tolerance test mice will be

fasted up to 6 hours and no more than two procedures will be performed on any individual animal. The effects of androgens on appetite and bioenergetics will be evaluated using metabolic home-cage monitoring for one week prior to microchip implantation as well as the final week of the experiment. At the end of the protocol cerebrospinal fluid will be collected for steroid analysis under deep non recovery anaesthesia. Androgen sensitive tissues (fat, ovary, choroid plexus and muscle) will be weighed and collected for analysis post mortem.

In addition we wish to explore the synergistic role of androgen excess and obesity in modulating intracranial pressure in mice. Therefore an additional cohort of mice will be raised on a high-fat diet (up to 60% kcal from fat).

What are the expected impacts and/or adverse effects for the animals during your project?

Home cage metabolic energy expenditure will be monitored at week -2 and week 6 for a duration of up to 7 days each. During the acclimatisation phase, animals have to settle into new surroundings, and get used to the food hoppers and water bottles in the system. Animals display cautious behaviour and may feed and drink slightly less than normal leading to weight loss <5% (incidence 100%).

Glucose tolerance test will take place at week -2, for a duration of 3 hours. Administration of glucose is unlikely to result in any adverse effects and is well tolerated.

At week -1 animals will be anaesthetised for a surgical procedure that will approximately take 60 minutes. This would entail implantation of an intracranial pressure microchip. This procedure should not result in adverse effects and is well tolerated. Animals will experience transient discomfort following the implantation after waking from anaesthesia (100% incidence) but will receive analgesia. The animals will be allowed to recover for a minimum time of 7 days.

At week 0, under anaesthesia, animals will undergo a second surgical procedure that will takes up to 30 minutes. This will involve the implantation of an androgen minipump. This procedure is also unlikely to result in adverse effects and is well tolerated. Animals will experience transient discomfort following the implantation after waking from anaesthesia (100% incidence) but will receive analgesia.

Blood collection from a superficial vessel should result in only subtle signs of adverse effects indicative of transient pain and stress due to pin-prick and handling.

A group of mice will be fed a high-fat diet for 6 weeks. Animals receiving high fat commercially available laboratory chow have not been associated with any physical or behavioural abnormality, but the coats of the animals will become greasy and excessive grooming may occur, resulting in skin irritation.

Oestrus cycle monitoring will take place one week following androgen and microchip implantation surgery and will take 5 minutes per swab (swabs will occur on three days during this week). Animals will be weighed weekly. An assessment of intracranial pressure

will take place twice weekly in freely moving mice for 3 hours for up to 6 weeks. Animals will experience stress due to initial restraint.

Cerebrospinal fluid collection will occur at the end of the experiment under non recovery anaesthesia before humane killing and tissue collection post mortem.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Expected severities of procedures include: Metabolic monitoring (mild); glucose tolerance test (mild);Implantation of intracranial pressure microchip (moderate), insertion of minipump (moderate); blood sampling (mild); administration of a high-fat diet (mild); oestrus cycle and weight monitoring (mild); intracranial pressure monitoring (mild).
- When summarising the expected severities of all procedures, the cumulative severity will be moderate for 100% of the 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?

Raised intracranial pressure occurs only in the intact living brain due to increased cerebrospinal fluid secretion from the choroid plexuses in vivo within the brain ventricles with intact cellular and vascular architecture. Intracranial pressure cannot be reproduced in vitro, and not enough is known about idiopathic intracranial hypertension to construct computer models.

Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially adopted in order to replace in vivo animal use.

Which non-animal alternatives did you consider for use in this project?

Our existing in vitro data obtained from cell culture approaches has guided the proposed in vivo studies. We always conduct cell culture based experiments to justify the need to use animals. These systems include culturing cells in monolayer as a homogeneous population on plastic or mouse/human derived tissue as organoids.

Why were they not suitable?

There are significant limitations of basic culture conditions using cells gown in isolation as a monolayer on a piece of plastic as this is not an accurate representation of what occurs within a patient. There is an inability to fully recapitulate the in vivo 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?

When possible we will always use power calculations to determine estimated numbers of animals that should be used. For most quantitative experiments, animal cohort size will be calculated via power analysis. Expected effect size will be determined through consultation of the literature, cell culture based in vitro analysis or through small pilot experiments when possible (have been previously conducted by our collaborators).

We have used statistical methods to calculate how many animals we need to get meaningful data.

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 to design experiments and taken advice from researchers who have performed similar research projects in the past.

We aim to reduce animal numbers, apply methods to reduce subjective bias, and undertake appropriate statistical analysis without compromising the scientific objectives. When possible, experiments will involve a factorial design that will maximise the information obtained from a minimal number of animals.

For example, we will only conduct the experiments in one species (mice). Mice raised on a normal diet with androgens and a further group without androgens will serve as a control for mice raised on a high- fat diet.

We have also been able to accurately assess the variability of intracranial pressure measurements and thus the standard deviation used in the power calculation using our existing data collected with our collaborators.

By using a minimally invasive technique the procedure is less severe with less harm to the animal compared to an invasive intracranial pressure monitor. This will enable fewer mice to be used 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 have used previous pilot studies to estimate variability and perform power calculations to calculate sample sizes.

Prior to all experiments we will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment. The resulting data will be published in Open Access Journals wherever possible and in accordance with the ARRIVE guidelines.

All tissue surplus to requirement will be stored and made available to collaborators within the institution and beyond.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used for implantation of the microchips. Subcutaneous implantation of androgen minipump for prolonged release of dihydrotestosterone during the course of the experiment will be undertaken after a minimum of 7 days post microchip surgery. The intracranial pressure will be recorded in freely moving mice for a total of 6 weeks.

The androgen minipump implantation is a well-known technique and an established skill set within the animal facility. Previous studies have shown no known prolonged discomfort in these animals. This is a refinement to a daily injection of androgens which has been conducted in the past.

The intracranial pressure microchip implantation is minimally invasive compared to traditional intracranial pressure monitoring using a probe into the brain. This is a significant refinement as this technique involves drilling a smaller burr hole (0.5mm diameter) without penetrating the dura, therefore reducing the pain, suffering and distress to the animals whilst also delivering highly accurate data.

To measure the energy metabolism of mice in vivo we employ metabolic home cage monitoring. Unlike other metabolic cage systems, this approach allows mice to live in their home cages, and so CO2 production, O2 consumption as well as food and drink intake can be monitored without any disturbance to the animals.

Why can’t you use animals that are less sentient?

Mice are the least sentient species that will allow us to achieve our objectives.

We have chosen to use mice over other less sentient species as mice and humans share 97.5% of their coding DNA sequences. Moreover they are the ideal size due to the nature of the experiments in terms of implantation and assessment of responses.

In vitro studies cannot fully provide essential information about raised intracranial pressure that is obtained from in vivo studies, because the complete physiological response to raised intracranial pressure involves a closed system of pressure build, such that valid treatment testing requires the study of intracranial pressure in vivo.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is a key consideration in all of our protocols and we will be guided by our NACWO and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare Animal welfare is a key consideration in all of our protocols and we will be guided by our experienced technical and veterinary staff in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local animal welfare and ethical review committee. Following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

Analgesic will be administered at the time of each separate surgery for intracranial pressure microchip and androgen minipump implantation both during the procedure and approximately 24 hours later for post-operative analgesia. The mice will be observed closely for any evidence of discomfort, and should we observe such evidence they will be humanely killed.

We will make every effort to group house the mice in between procedures.

We are fortunate to have excellent colleagues with extensive, relevant animal procedure experience, from whom we can learn refined techniques from.

Examples: The home cage metabolic monitoring system allows the quantification of energy metabolism by measuring O2 and CO2 concentrations, food and drink intake while the animal is living in an individually ventilated cage without any disturbance. For glucose tolerance testing a single incision will be made on the tail to take small sequential blood samples rather than sequential cutting for blood sampling from tail vein. We use ultrasensitive ELISA assays for hormone measurements to reduce the blood volume that has to be taken. Refined handling techniques are used wherever possible and body condition charts for humane endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the literature for experimental design, best practice, and humane endpoints for research in animals.

Prior to all experiments we will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment. The data will be published in Open Access journals that support the ARRIVE guidelines, published by the NC3R's, and conduct our experiments with advice from the PREPARE publication (PREPARE: guidelines for planning animal research and testing. Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017 Aug 3.PMID: 28771074).

The LASA guidelines: 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.)

Jones HRP, Oates J, Trussel l BA (1999) An applied approach to assessment of severity. In: Humane End points in Animal Experiments for Biomedical Research (Hendriksen CFM, Morton DB, eds).

London: Royal Society of Medicine Press, pp 40±7

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Literature searches, attendance at vendor's information sessions, seminars and conferences to find out about new technology and new approaches that we could implement.

We will comply with the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments; www.nc3rs.org.uk/arrive), an NC3Rs-developed checklist of the essential information that should be included in publications reporting animal research.

We will consult the NC3Rs social media platforms to help us keep up with advances in the 3Rs which will be discussed an implemented through our lab meetings.

51. Understanding glaucoma pathology and the development of new therapeutic strategies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neuroprotection, Retinal disease, Glaucoma, Fibrosis, Drug delivery

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this programme of work is to understand how and why glaucoma develops and to investigate if we can treat the underlying causes of glaucoma with new experimental therapies.

Glaucoma is a complex disease that leads to blindness and currently there are no curative therapies. In this programme of work we aim to understand and treat the causes of glaucoma by investigating cells, fluids and tissues within the front and back of the eye.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue **after the project has finished.**

Why is it important to undertake this work?

Glaucoma is a neurodegenerative disease and is the leading cause of irreversible blindness worldwide. There are approximately 80 million people affected by glaucoma with

11 million people worldwide being bilaterally blind from the disease (https://www.iapb.org/wsd14-case-glaucoma/).

Currently there are no curative therapies available for glaucoma and patients lose their sight over time.

What outputs do you think you will see at the end of this project?

The main outputs from this project will be:

Improvements in understanding how glaucoma develops and cellular and extracellular disease processes that occur within the front and the back of the eye. Furthermore, we will have gained knowledge in understanding the role(s) of fibrosis in the development of glaucoma.

The development and assessment of novel therapies to treat disease at the front and back of the eye in glaucoma.

The development and assessment of ocular drug delivery technologies in order to treat front and back of the eye pathology in glaucoma.

The knowledge and data generated through this project will be disseminated through patient and public events, conferences, high impact peer reviewed publications and/or future grant applications.

Who or what will benefit from these outputs, and how?

Being able to demonstrate preclinical efficacy of potential therapeutic agents will enhance their translation into the clinic, and therefore this research has the potential to significantly reduce patient, clinical and economic burdens associated with glaucoma. In the UK over 2% of people over 40 years of age have glaucoma. Sight loss due to glaucoma poses significant emotional and physical burdens on patients.

Short-term (during the project): within the five year programme, beneficiaries will be the local, national and international research communities involved in glaucoma, fibrosis and drug delivery research. Benefits will include both scientific discovery and technical expertise, which can be shared through social media, conferences, meetings and publications. Also in the shorter term, patients will be made aware of the work through glaucoma and ocular disease patient groups (for which our team is already part of).

Long-term (after this project): Through knowledge acquisition and therapeutic discovery, patients and clinicians will be the most likely beneficiaries to this research. For patients, it will be the development of a treatment which offers anti-scarring, pro-regenerative and neuroprotective therapies, administered in a way that is patient acceptable and reduces side effects of current IOP lowering medications. For clinicians, the development of disease modifying therapies would be revolutionary for glaucoma.

Furthermore, being able to administer therapies in a manner suitable to ensure patient adherence to treatment regimens would be of benefit to the clinical community and patients.

How will you look to maximise the outputs of this work?

The outputs of this research will be maximised at an Establishment level by the sharing of surplus animal tissue, and at national and international levels by promoting collaborations, the dissemination of new knowledge, and the publication of results and methods to inform the scientific community.

Surplus animal tissue: given that a lot of the research will be based on the eye and central nervous system, other surplus animal tissues from these experiments will be freely available to other researchers.

Collaborations: we have recently set up collaborations to investigate mechanisms of TM/SC dynamics. This project would permit knowledge sharing and allow us to develop improved ex vivo modelling to further reduce the need for in vivo experiments.

Knowledge sharing of positive and negative results and approaches: results from this project will be important for advancing our knowledge of glaucoma pathology, new disease targets and any new treatments. The results of the models, the techniques and data will be published in peer reviewed journals (ophthalmology, general medical, drug delivery and method journals) in order to reach a wider audience. Furthermore, social media will be used to promote the research and its results. It is important that any research pitfalls or challenges be addressed and described in order to allow others to learn and optimise methods.

Species and numbers of animals expected to be used

- 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.

Rat models of glaucoma 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. Glaucoma typically effects adults and therefore adult rats have been chosen for this project.

Typically, what will be done to an animal used in your project?

Typically, under a general anaesthetic, rats will have their eye pressure non-invasively measured (using a probe that gently touches the cornea to calculate the pressure inside the eye) and then have glaucoma induced by a small injection into the front of their eye under a general anaesthetic. These injections are given twice a week for 1 month. Usually at around two weeks from the start of the experiment, rats will be given a treatment, either as an eye drop or as injections into the eye to treat their glaucoma. All of the methods used here are routinely performed on humans with glaucoma in a clinical setting (although

humans receive a local rather than general anaesthetic). Rats may also have non-invasive imaging of their eyes performed at the end of the experiment using optical coherence tomography (the same type of imaging which people receive at the opticians). The experiments will typically last for 1 month and rats will have a maximum of 9 anaesthetic interventions.

What are the expected impacts and/or adverse effects for the animals during your project?

Glaucoma doesn't present with symptoms in humans, and it's one of the main reasons why the irreversible sight loss is so profound. It is also one of the main reasons why patients forget to administer their own eye drop treatments. As with humans, the overall presence of glaucoma in the rats will not induce any adverse effects or pain for the animal. The rats will experience transient adverse effects from the multiple intraocular injections which could lead to some discomfort of the eye, however given that the rat will be under a general anaesthetic, any eye irritation will have likely worn off when the animal recovers. Topical analgesia may however be given as a precautionary measure if required.

Typically the injections take 3-5 minutes each. For the treatments, although unlikely, there may be intolerance to the therapeutic agent, however, as most agents will be given locally to the eye, this is not expected to reach the systemic system and therefore the risk of toxicity or intolerance will be very low. For the assessment of new therapies, all rats will be closely monitored for any weight loss or abnormal behaviours. Additionally, all new therapeutic agents will have undergone extensive testing via other methods prior to use in living animals to minimise any risk of adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The protocols have been classified as moderate due to the repeated injections into the eye and the repeated use of anaesthetics.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 substitute for the in vivo models we plan to use. We need a living system in which to accurately mimic the human condition of glaucoma so we can understand and study the complex interactions and functioning of the fluid systems and drainage tissues

within the eye and how they control internal eye pressure. We also need a fully functioning living system to provide a scenario in which we can manipulate eye pressure and investigate the downstream complex signalling and release of factors which lead to retinal and optic nerve degeneration - the cardinal features of glaucoma in humans. Without the in vivo system, we would also be unable to investigate and identify treatments that interact with the multiple responsive cell types involved in glaucoma, together with the immune and inflammatory response signals which cannot be modelled in vitro. Whilst it is unethical to experiment on human subjects to model glaucoma, we will also be using waste tissue from human surgeries to help inform the project.

Which non-animal alternatives did you consider for use in this project?

We have and continue to use the culture models of the particular cell types involved and these can give us a good idea of how those cell types behave in certain contexts, however, they are unable to model the complex interactions between the fluid production, drainage and pressure in the eye, and how all of this can lead to retinal and optic nerve damage. Furthermore we have considered the use of porcine (waste tissue) and human donor eyes as surrogates to try and understand how we can deliver drugs into the eye.

Why were they not suitable?

Culture models or ex vivo eyes are unsuitable for modelling glaucoma. Cultures are not currently complex cell systems which can interact with all the cell types involved in glaucoma. Ex vivo porcine or human eyes are not living tissues, and as such they cannot be used as glaucoma models because they cannot be maintained for the length of time required to develop glaucoma and assess the therapeutic potential of new treatments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers estimated in this project are based upon 8 years of experience of experimental design and from previous experience of using this same model. Most of the experiments have quantitative end points, and previous experiments have demonstrated that we require around 8-9 animals per treatment group in order to develop statistically sound results. Although we reduce the variability as much as possible, some rats have innate differences in their eye pressures and/or may not develop glaucoma. We know from past experiments that generally >6 out of 10 rats will respond to the induction of glaucoma, therefore, this has been taken into account when deciding on the total numbers for the project. In addition, reduction in animal numbers will be achieved through the use of both eyes to induce glaucoma, therefore if only one eye develops high intraocular pressure then that animal can still be used in the experiment reducing the need to use more

animals. The use of both eyes also enables eye tissues and fluids to be pooled for analysis where needed. For qualitative end points less animal numbers will be used, typically 3-6 animals per treatment group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Based on previous studies, randomised designs are used for most experiments where animals are randomly assigned to a treatment arm. Studies will usually monitor the effects of therapies to lower eye pressure throughout the experiment. Most of the experiments are hypothesis driven with clear end points to help aid the design. Study designs are checked against the ARRIVE2.0 guidelines and the EDA tool will be used in order to minimise any error in design or bias and to ensure we report the study results comprehensively. Furthermore, 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?

Initially studies (modelling the disease and doses of therapies) will be designed based on previous experiments. Given that we have over 8 years' experience of using this model, we are confident in the experimental design for the induction of glaucoma. The key optimisation steps are therefore around testing of the therapeutics. Most of our therapies have been administered by topical or injectable routes. So for similar compounds our experimental design protocols are already in place. However, we are developing novel ways in which to enhance drug delivery (use of hydrogels, cell penetrating agents) and so we would perform pilot studies for the new compounds to enable us to establish a therapeutic window in which to use these compounds in the larger glaucoma studies.

We will optimise the data and information from all animals in all experiments. Furthermore, unused tissues from experimental or control animals will be made freely available to other scientists within the University.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 rats. They have been rigorously characterised and are suitable for use in glaucoma as they possess comparable eye and visual systems, develop the same clinical signs (e.g. ocular hypertension, trabecular

meshwork scarring and retinal degeneration) and have the same molecular and cellular signalling cascades that lead to glaucoma in humans. This glaucoma model has been further refined from the previously used optic nerve crush model. In the optic nerve crush model over 95% of retinal neurons are damaged, in this more refined model, there is only between 30-40% loss of retinal neurons and it is more representative of the human condition.

Furthermore, although the rat eye and visual system are identical to humans, rats do not rely on sight as their primary sense. Generally, the injections take less than 10 minutes to perform under general anaesthetic and the rats demonstrate a full return to normal behaviour within a few minutes of waking.

Additionally, whilst this procedure is not thought to cause postoperative pain, where appropriate topical analgesia will be applied at the time of the injection as a cautionary measure. We have taken care to develop the models to have minimal adverse effects on the rats and all the techniques employed in this project are routinely performed in humans in the clinic.

Why can’t you use animals that are less sentient?

Although mice also possess a similar eye structure and function to rats, they are not feasible for these experiments due to the very small size of the eye, making intraocular injections challenging. If mice were to be used, it would likely result in the use of more animals due to technical failures. Less sentient animals such as fish are not suitable for modelling human neurodegenerative diseases as, unlike mammals, they are able to regenerate their injured central nervous system.

How will you refine the procedures you’re using to minimise the welfare costs (harms) for the animals?

The animals will be allowed to acclimatise to their surroundings for 5 days and get used to being handled. Monitoring forms will be used for the rats and daily monitoring will be conducted especially for any new therapeutic interventions which could have unexpected effects on the animals. All experimental time points will be kept to a minimum and multiple analyses will be conducted from each animal during and after the experiment. The number of interventions will be kept to a minimum and the minimal drug volumes will be used. Topical analgesia will be used where appropriate as a cautionary measure. We are also putting refinements in place by determining the most efficient way to deliver drugs into the eye in the least invasive manner. 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.

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 courses provided by the Animals in Science Regulation Unit, United Kingdom and attended workshops on the ARRIVE guidelines (2017) and NC3Rs best practice in experimental design (2019). I have also attended online seminars including the NC3Rs' "improving the quality of funding applications using animals" in 2020. I intend to stay informed by attending local workshops and engaging in seminars and events given by the NC3Rs.

52. Engineering plant ion channels and receptors

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

plant physiology, ion channels, molecular engineering

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 goal of this project is to understand how ion channels work in plants in order to develop strategies for improving crops. Ion channels are proteins that facilitate the movement of ions across cell membranes and are vital for many functions in plants, including photosynthesis, resistance to pathogens, and environmental hardiness.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ion channels are important targets for research because of their potential for improving crops. Pioneering studies from research groups around the world over the past few years have provided proof-of-principle for enhancing crop biomass yields and water use efficiencies. For example, by accelerating ion fluxes, it has been possible to improve photosynthetic carbon fixation. These advances are important, because they demonstrate the potential to greatly increase crop production and, at the same time, to do so with a reduced cost to the environment.

We know a great deal about how the plant ion channels work, and we have knowledge sufficient to begin engineering their protein structure in order to carry out these improvements. What we do not yet know is which parts within these proteins are the best for engineering to achieve the improvements that are desired. For example, we know that altering key behaviours of certain channels will enhance the ability of plants to cope with environmental challenges such as drought; what we need to identify are the molecular parts that are best targeted for change in order to achieve these enhancements.

For this purpose, being able to characterise the plant channels when introduced into Xenopus eggs is vital, because it allows an analysis of the channels free of the background of regulatory factors that are present within the plant cell.

The immediate challenges now are

to identify the relevant molecular parts of each of a small subset of ion channels for engineering, and

to resolve the range of enhancements that can be achieved through such engineering for transfer back to crop plants.

What outputs do you think you will see at the end of this project?

The primary outputs of this work will be in proof-of-principle knowledge and in scientific publications. There is much interest currently in finding ways to enhance crop yields and their efficiencies in water use in the face of global environmental change. Thus, the research is expected to find its way into the highest calibre international journals. As a proof-of-principle, the project is likely also to yield one or more patents and to lead to research that will translate the findings to agricultural crops.

Who or what will benefit from these outputs, and how?

This project is directed to the related challenges of increasing biomass yield and reducing water consumption of crops, and it also addresses the development of tools that make use of biological 'switches'. The concepts behind the proposal are at the core of ideas emerging within the international plant biology communities. These approaches have already proven highly successful using synthetic proteins that have been introduced into many cell types. Using similar strategies that are built on naturally-occurring proteins is one of the most challenging problems in biology. Solving these problems is expected to yield fundamental knowledge as well as informing on ways to improve crop water use and yields.

Benefits

In short-term, the research will benefit fundamental science in its focus on new tools, methods and critical understanding about how plant ion channel proteins function. It will generate a number of molecular tools, based on study of the channels when isolated from the plant, that will enable researchers to probe and understand the physiological mechanics of plant cells and tissues. These tools will allow researchers approach questions that have hitherto been intractable to analysis. For example, it will be possible to

manipulate the mechanics of ion and water flux in real time in order to understand their coordination.

In the medium- to long-term the research it will benefit the applications of scientific knowledge in guiding future work to improve crops. In particular, once we know how to modulate the activity of the ion channels, it should be possible to develop crops with native channels that have been altered in their activity in order to improve both ion and water flux efficiencies, thereby benefitting farmers and society in general by reducing the resource demand of growing crops.

With regard to the applications of this knowledge, it is worth noting that, at present, agriculture consumes 70% of our natural fresh water resources globally. Even in the UK, irrigation of crops has expanded some 1000% over the past 30 years in the face of environmental changes we are experiencing. This trend is set to continue will soon be unsustainable.

How will you look to maximise the outputs of this work?

The project is certain to lead to several platforms for dissemination of the results, including opportunities to share the knowledge with academic and industrial researchers through seminars at scientific meetings, through publication, and through sharing of biological materials, including molecular constructs, with academic colleagues for their own research.

Species and numbers of animals expected to be used

- 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.

Toad (Xenopus) eggs are essential for the data collection element of the studies that use electrical recording methods, so-called electrophysiology, to measure the functional characteristics ion channels. These elements data collection need to be carried out by introducing the ion channels into a non-plant cell in order to characterise the channel behaviour free of interference from regulatory factors that are present in the plant cell. As a further complication, such recordings require data collection over longer periods for full characterisation of plant channels. Such long-term recording periods can only be achieved in Xenopus eggs; they cannot be achieved in other non-plant systems such as mammalian cell (eg. HEK cells) and insect cell lines (eg. Sf9 and Sf21 cells) in which only short recording times are possible. Thus, analysis of channel behaviour initially requires that the channel proteins are situated in a Xenopus egg.

For these reasons, recording from Xenopus eggs has been the benchmark for studies of this kind for over 30 years. These measurements will be important for comparative

purposes and will lead thereafter to further analysis in the plant. For proper introduction of ion channel proteins, it is essential to use eggs at stage VI (late in development), as the eggs become dormant following release and will no longer produce ion channel proteins. Eggs will be harvested by surgery and partial removal of the ovaries of adult female Xenopus toads.

Typically, what will be done to an animal used in your project?

The use of toads (Xenopus) will be restricted to the harvest of unfertilized eggs. An egg harvest will entail one surgery under general anesthesia to removal of part of an ovary with recovery. The total number of animals will be limited to the minimum needed to provide essential insights that can be transferred to analysis of the ion channels in the plant.

What are the expected impacts and/or adverse effects for the animals during your project?

Possible adverse effects include post operative infection and wound breakdown. These are rare events

- the experience of post-operative infection is less than 0.1% over the past two decades - as measures are in place to minimize the risks to the animals and antibiotics may be used to eliminate such infections, usually within 1-2 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of the operation is moderate and measures are in place to minimize the risks to the animals. All animals will be subject to this operation severity. Each animal will undergo a single operation under anesthetic with recovery to harvest a collection of eggs. A second egg harvest will be carried out after killing the animal by a Schedule 1 method.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Use of toad (Xenopus) eggs is essential for a subset of data collection. Analysis of function requires that the proteins are introduced into a non-plant cell, since the plant cell will have these channels and related proteins, making defined analysis impossible.

By carrying out these elements of data collection after introducing the channels into the eggs, it will be possible to characterise the channel behaviour free of interference from regulatory factors that are present in the plant cell. Once the characteristics of the channel protein have been determined in this way,

it will then be possible to re-introduce the channels into the plant and to assess the consequences in the native situation.

Which non-animal alternatives did you consider for use in this project?

Mammalian cell lines (eg. HEK293 and CHK cell lines) and insect (eg. Sf9 and Sf21) cells were considered. Recordings from these cells are not possible for two main reasons that are outlined below.

Why were they not suitable?

For analysis of many ion channels, it is necessary to co-introduce 3-5 different ancilliary proteins at the same time; the success of such co-introductions in both mammalian and insect cell lines is vanishingly small, making any comparative analysis impossible

The characteristics of many ion channels demand that electrical recordings are extended over long time periods, often many tens of minutes to hours; such long recording periods are simply not possible with mammalian and insect cell lines. Such stable recordings are essential to analyse many of the properties of the ion channels before they can be transferred to any plant 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?

Numbers of animals are estimated based on previous projects with similar requirements for testing using toad (Xenopus) eggs.

In general, the major limitation on use of toad eggs is the time over which they survive following an operation and may express proteins. Once isolated, the eggs survive for only 5-8 days and, after a protein has been introduced, there is typically a 2-3 day period over which measurements can be made. The second major limitation is the number or measurements that can be made during this 2- 3-day period. A trained researcher will be able to carry out 6-8 measurements in the course of one day. Finally, the third limitation relates to the quality of the eggs themselves, which will vary from one animal to the next. For this reason, to fully analyse any one protein, it is standard practice to obtain at least 4-5 measurements from each of at least three separate egg harvests.

Because each egg harvest typically yields some 100 usable eggs (ie. in stage VI), significant savings can be achieved by careful planning so that 4-5 different proteins are analysed with the eggs from each harvest.

The need to analyse some 70 different proteins is anticipated over the course of this project. Assuming that roughly 10% of toads do not yield viable stage VI eggs, the above figures indicate the need for 40 animals over the during of the research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental designs take into account opportunities to reduce animal numbers by subdividing each egg harvest into pools and planning experiments to address parallel questions concurrently. This approach is possible as each harvest normally yields more eggs than are needed for a single experimental trial. Following a harvest, the eggs are normally viable for a period of only 5-8 days. Extra eggs from a harvest cannot be stored for longer times, for example by freezing, because the egg membrane breaks down which renders them unusable for recordings. Separate pools will be used therefore to express and test different protein constructs or construct combinations in parallel, thus reducing the total number of animals required.

Undertaking surgery with recovery also reduces the number of animals to be used. The quality of Xenopus eggs varies from one animal to the next and is generally consistent within any one animal. Recovery surgery therefore allows the selection on the next egg harvest of a single animal needed to obtain good eggs after Schedule 1 killing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Measurements of the activities of the ion channels will build on pre-defined protocols that are the gold standard for channel analysis and will include the parallel experimental designs noted above.

Additionally, these designs will be guided by established computer modelling of the electrical current characteristics and, where possible, by modelling with known physical structures of related ion channels. Electrical currents will be compared with those obtained from wild-type channels (these

include measurements of temporal kinetics, voltage sensitivity, inactivation kinetics, ionic dependencies, etc.) and will be deemed satisfactory for any one construct using normal statistical standards.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Use of toad (Xenopus) eggs, as noted previously, is important for introducing in a non-plant cell and analysing the ion channels, and is the benchmark for studies of this kind. To minimize suffering, a single partial removal of an ovary will be carried out under anesthesia with recovery. A second ovary removal will be carried out only after the animal killed by a Schedule 1 method.

Why can't you use animals that are less sentient?

For proper expression it is essential to use eggs at stage VI (late in development) harvested from toad (Xenopus) ovaries, as the eggs become dormant following release and can no longer be used to introduce proteins for analysis. The eggs are large, easily handled, and enable long-term recordings that are essential for the type of analysis needed to characterize the plant ion channels and their characteristics.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At present, care includes the isolation and close monitoring of each animal after operation until normal behaviours and feeding resume, typically within 12-24 hours. refinements will include the use of local analgesics, subject to guidance from the NVS.

Otherwise, knowledge transfer to work with in relevant native plant systems will be sought as quickly as possible, consistent with groundwork established through analysis of the ion channels when introduced into the eggs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal husbandry and surgeries will follow best-practice guidance from NC3R and the RSPCA to ensure that egg harvests are as refined as possible.

For experiments using the Xenopus eggs, measurements of the ion channels will use established protocols that are standard for research of this kind (e.g. two-electrode voltage clamp and patch clamp methods). Comparisons with known channel characteristics and internal controls, including quantifying the amounts of ion channel protein present, combined with statistical standards (T-test, ANOVA) will ensure maximum refinement of post-operative experimental procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am updated through the designated office of relevant advances and seek to implement these in consultation with the NVS and NACWO. I expect to take part in 3R events organised within the the establishment I also keep up with the relevant literature and keep informed through regular attendance at conferences.

53. The effects of myocardial infarction.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Cardiology, Myocardial infarction, Ischaemia, Ventricular fibrillation

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 project aims are to investigate the electrical and mechanical alterations that occur during the different stages of recovery following a heart attack in both the surviving and damaged regions of the heart.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue **after the project has finished.**

Why is it important to undertake this work?

In man, heart disease is common, with a poor prognosis. The underlying mechanisms are complex; in some cases, there is a steady deterioration and in others the terminal event is sudden and unexpected. Current treatments are unpredictable in their effectiveness and often disappointing in their ability to reduce mortality. There are examples of studies that

have had to be terminated early due to an increase in mortality in the treatment groups. As these treatments were primarily studied in healthy hearts there is a possibility their effects are different in diseased hearts.

Many patients with diseased hearts have arrhythmias (abnormal heartbeats) and some treatments may have secondary effects at a cellular level that may further compromise the heart's ability to function properly. By far the most common cause of heart failure is myocardial infarction, i.e. ischaemia to myocardium caused by chronic arterial artery disease.

This project will use animal models of myocardial ischaemic damage. In our laboratory, a coronary artery will be permanently or temporarily tied off giving a small area of damaged tissue. The heart will then be studied whole or single cells will be used to examine mechanical and electrical changes. We are also developing a less invasive procedure which will reduce side effects and create an infarct more similar to that seen in humans. Rather than using surgery, a wire will be guided from an artery in a leg or arm into the arteries that supply blood to the heart and an artery will be blocked to simulate a heart attack. This procedure will reduce the risk of post-operative effect.

Studies on human hearts are difficult for several reasons including difficulty in accessing both diseased and normal tissue. Variations in age, medication and other health problems make it difficult to reliably investigate the processes at well-defined time points after a single incidence of damage.

The research proposed using this model is designed to provide new insights into the electrical events that trigger and sustain arrhythmias in cardiac muscle. An improved understanding of what causes these arrhythmias and their relationship with reduced function in a damaged heart is necessary to develop successful treatments that maximise heart performance while minimising the risk of life- threatening arrhythmias. Additionally, the models are used to investigate novel methods designed to improve the strength of contraction after an MI by adding a patch of artificial myocardium.

What outputs do you think you will see at the end of this project?

The project will provide information about the causal mechanisms that link heart tissue damage that results from a block of the coronary artery to (arrhythmic) sudden death. It will provide basic knowledge of the physiological and pathophysiological processes that occur post infarct and will inform future research so we may better understand the hearts healing process and suggest alternative therapeutic approaches. In the clinic, the currently available drugs, although having a rational theoretical basis, have been unpredictable in their clinical effects and have often been particularly disappointing or only marginally effective in their ability to reduce mortality. Indeed, there are numerous examples of trials that have had to be terminated early because of evidence of increases in mortality in the treatment groups.

One possible explanation for these adverse poor effects is the lack of understanding of the underlying primary cause of the electrical disturbances that lead to sudden arrhythmias at the different stages post- infarct. For example, there is increasing evidence that many

antiarrhythmic drugs have different electrophysiological effects in abnormal myocardium that could indeed exacerbate the propensity for arrhythmogenesis. These drugs have primarily been studied in isolated normal tissues whereas arrhythmias electrical disturbances presumably usually arise in damaged a scarred myocardium. It is therefore hoped that the findings of this study will inform subsequent work designed to tailor therapies to the various types of scars that emerge after an area has been damaged by block of the coronary artery. If a new potential therapeutic target or that a currently available drug may have beneficial effects at a given stage post infarct, subsequent studies will incorporate appropriate treatment groups in order to examine that rationale. The results from these studies will provide information concerning the structural and molecular basis for the changing predisposition to arrhythmias and sudden death post- MI, as well as information concerning contractile dysfunction. The work will be published in peer- reviewed journals and allow scientists to develop biomarkers that give an early warning of the arrythmias that lead to sudden death. Furthermore, the knowledge of mechanisms will allow drugs to be designed that will reduce the vulnerability of people with scarred hearts to cardiac arrythmias.

Recent developments in cell biology have resulted in the production of artificial myocardium heart muscle from somatic cells induced into a pluripotent form before differentiation to cultured cardiac cells. These cells can be used to manufacture myocardial patches of artificial heart muscle that can be embedded in the scar and supplement the few surviving myocardium strands of heart muscle post-MI. The new field of regenerative medicine has resulted from this work, but considerable research is required to develop viable patches that supplement myocardial mechanics without enhancing arrhythmias. This topic requires research using larger mammal hearts such as the rabbit with clinically relevant damage associated with the MI procedure to establish conditions that are optimal before testing on large mammals and man. In summary, the research described in this licence will aid in the modification of current treatment strategies to tailor new treatments to the stage of the remodelling process to repair and stabilise a damaged heart

Who or what will benefit from these outputs, and how?

In the short-term, the data gained from these studies will be published in high impact journals, adding to the body of knowledge concerning the mechanisms underlying the adverse effects of heart disease.

This work will also test new therapeutic approaches designed to improve the outcome of patients with ischaemic heart disease. Once these projects have been completed, the approaches that have been established as effective in the animal model, will go onto be tested in human patients by clinical teams.

How will you look to maximise the outputs of this work?

We will continue to collaborate with national and international colleagues and disseminate this work through publication and social media at the earliest opportunity.

Species and numbers of animals expected to be used

- Rabbits: 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.

Rabbit hearts and those of larger mammals use electrical signals that have clear regional electrical variation that closely resembles that seen in the human myocardium. Furthermore, the structure of scars that result from damage to human heart tissue and those seen in the scars that develop in the rabbit heart are largely comparable making this species suitable for studying ways to stabilise and repair the human heart. Smaller mammals have very different electrical signals and the scars resulting from tissue damage are very different from the human heart. The rabbits used are approximately 6-8 months old, this is equivalent to a young adult human and therefore the heart structure is that of an adult animal and allows us to draw conclusions relevant to an adult human heart.

Typically, what will be done to an animal used in your project?

Most animals will only experience transient suffering while being administered an injectable anaesthetic for a terminal procedure (protocol 2).

The animals undergoing thoracotomy procedures will experience moderate pain from the surgery, which will be minimised by an analgesic regimen post-operatively. Further procedures on these animals will typically be limited to non-invasive imaging such as ultrasound and MRI measurements. Where other invasive procedures are required they will, where possible, be carried out under a final general anaesthetic.

Animals undergoing the new non-surgical procedure designed to block the coronary artery would will experience only mild pain associated with the use of cannulas.

All of the above animals would be expected to be killed around 8 weeks after coronary artery occlusion. The rabbits are not expected to show clinical signs of heart failure at this time point. Many humans feel no pain associated with acute coronary artery occlusion, some experience short-term referred pain, but long-term pain associated with a healing or scarred myocardium is not reported and therefore not expected. Any pain associated with the acute MI will be diminished by the post procedure analgesic regimen. Animals will be monitored for signs of pain or discomfort over the subsequent 8 week period and analgesia administered if clinical signs present. Past experience of thoracotomy-based MI procedure is that on recovery from the surgery, the animal does not show any clinical signs of pain or discomfort.

Animals undergoing stem cell implants of artificial heart muscle would experience moderate pain from the surgery. They would require daily injections of drugs necessary to prevent rejection of the implanted material and although the pain is transient, as it is a daily regimen, the procedural burden would be moderate. These animals would typically be killed no more than 2 to 4 weeks after the implantation procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will experience moderate post-operative pain which will be controlled with pain killers this effect typically lasts 5-7 days post-operation (100% of animals after a thoracotomy or a percutaneous procedure).

A small number of post-operative experimental animals (approx. 4% of MI procedures) will experience sudden and fatal arrhythmias. This is an unavoidable consequence of the model used and we cannot predict which animals will be affected.

Due to good surgical and husbandry techniques other adverse effects are very 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)?

Protocol 1 (P1): Surgical induction of MI- moderate severity - 19% of all rabbits. Protocol 2 (P2): Organ removal- mild severity 62.5% of all rabbits.

Protocol 3 (P3): Invivo cardiac phenotyping - mild severity 2.5% of all rabbits. Protocol 4 (P4): Percutaneous induction of MI - moderate severity 9% of all rabbits.

Protocol 5 (P5): Insertion of artificial heart tissue - moderate severity 7.5% of all rabbits.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

These studies require the use of animals rather than humans because the research concerns the behaviour of the whole heart using techniques that include some that can only be used on isolated hearts removed from the body while beating and maintained in specialised equipment for several hours.

Obtaining human hearts under these circumstances is only possible under very rare and special circumstances such as to make this practically impossible. Even access to samples of human myocardium is not practical since samples would be required of diseased and normal muscle. Limited surgical samples of small parts of ventricle (less than 0.1g weight) can be obtained during routine cardiac surgery of diseased but not normal tissue. The small size of the sample, the variation in site of origin, stage of disease,

age of the patient, existing medication and other co-morbidities makes it impossible to get consistent functional data on the parameters required for the study.

Furthermore, the particular projects described in this application are designed to investigate the behaviour of heart muscle in the context of the whole heart e.g. electrical activity of the scar in the context of the whole left ventricle using specialised imaging techniques. These measurements cannot be obtained from the small samples of tissue available from humans. Furthermore, the studies require measurements to be made at well-defined time points after a single incidence of damage. This level of consistency can not be achieved from human studies where the time of the initial coronary occlusion is frequently not known.

Thus, animal models are necessary to study both whole heart and cellular changes of heart muscle in response to damage and after the complex natural recovery processes have been completed. The same arguments applies to research on the effectiveness of artificial myocardium, currently being investigated as a means of repairing hearts. These initial trials would not be ethical or practical on human hearts. Once the new therapeutic strategies have been developed and refined and proven effective in animals, trials will be allowed in humans.

Which non-animal alternatives did you consider for use in this project?

The use of experiments involving cell culture were considered. There are commercially available sources of induced pluripotent stem cell derived cardiac muscle. This artificial heart muscle is considered suitable for attempts to repair the heart, but is insufficient by itself to mimic the complex process that occur during ischaemia and cardiac scar formation. There are no non-animal alternatives that duplicate the damage associated with block of the blood vessels in the heart and the subsequent processes involved in the repair of the heart muscle. Working on isolated hearts from an inbred animal line after a standardised procedure to induce scars will allow us to better understand the events that lead to electrical disturbances and sudden death. These processes cannot be replicated in cultured cells systems.

Why were they not suitable?

There are no cell culture alternative because the process of coronary artery blockade, cell death and then the repair process involving the formation of a scar cannot be duplication outside an animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used this established model of damage to the heart from a blocked coronary artery for a number of years. Based on previous experiments supported by past Project Licences, we know the inter-measurement variation and the size of effects that are functionally relevant. This has allowed us to calculate the sample sizes needed to carry out the experiments and the further proposed studies outlined in this application.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure the minimum number of animals will be used in each section of the project, the experiments have been designed using power calculations to establish the minimum number of animals required to obtain statistical significance between variables. In general, for each measurement, a group sizes of between 8-10 animals will be required. If we design experiments that appear to require greater than 12 animals to achieve the statistical significance, we redesign the experiment/measurement to ensure a group size of 8-10 animals is sufficient

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To optimise the animals used in the study, we continually analyse and test statistical significance as the study progresses; when differences do emerge at the 5% level by n=8, that aspect of the study is stopped, i.e. we do not use more animals for each sub-project than is necessary. If statistical significance is not achieved (or very close to the 5% level) by n=8, the particular measurement is not continued.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging Refinement techniques during the lifetime of the project.

Which animal models and methods 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 1 and 4 have been developed to model a common heart malfunction, described in this project which develops secondary to a block of a blood vessel in the heart and subsequent muscle damage.

Protocol 1 involves a long established method of a single episode of surgery to induce the myocardial injury. We aim to minimise the associated adverse effects by careful anaesthetic and surgical technique, carried out by experienced staff; the use of pre-emptive, intra- and post-operative analgesia, in consultation with the veterinary staff; detailed post-operative monitoring of animals and rapid intervention in cases of post-operative complications.

Previous experience with this model indicates that the expected adverse effects can be controlled and minimised.

In protocol 4, we are developing a non-surgical method of coronary artery block which will reduce or eliminate many of the adverse side effects associated with the surgical model and will provide experimental tissue with damage more similar to that which is seen in humans.

In protocol 5, the use of artificial heart tissue will be tested with a single surgical intervention. Experienced licensees will perform the surgical techniques under aseptic conditions and appropriate pain relief will always be administered. Best practice post-operative care will be employed to ensure any discomfort is minimised. Regular examination by veterinary surgeons and experienced technicians will ensure that any untoward effects that do develop are detected early and steps taken to minimise any distress or discomfort.

Why can't you use animals that are less sentient?

The rabbit heart has clear regional electrical variation that closely resembles that seen in the human myocardium. Furthermore, the structure of mature (healed) human infarcts and those seen in the rabbit are very similar making this species suitable for this study.

Smaller mammals do not develop such a human-like scar. This process of repair takes many weeks after the initial block of the blood vessel and therefore cannot be done as a terminal procedure under anaesthesia. Furthermore, the pattern of electrical disturbances that can be lethal in the human, also occur in the rabbit and involve electrical activity within the scar. This type of interaction is not properly mimicked by smaller mammals because of the size of the heart.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise the harms to animals that accompany our research, we have a long term plan to develop a percutaneous procedure (protocol 4) that will generate an MI and subsequent scar without the need for a thoracotomy. This will reduce the tissue trauma associated with the MI and therefore reduce the harm to the animal. It is hoped that this procedure can replace many of the thoracotomy-based MI procedures required for our research.

To minimise harms in the short term, we use pre and post-operative analgesia and monitor the animals for every 60 mins for the first 3 hours after surgery and twice/day for the first 3 days after surgery and daily thereafter. Robust local standard operating procedures for animal care, monitoring and minimal handling are in place. Lab members will ensure that all animals receive the highest standard of care to ensure animal suffering is kept to a minimum.

For each surgical procedure a Post-Op assessment sheet will be used (see image below) that will record the status of every animal with scoring and well defined limits and actions.

This will ensure that post-operative clinical signs including pain is recorded and appropriate well defined actions taken. This document is referred to at the appropriate places in the Protocols.

Rabbit Post-op monitoring sheet

Rabbit I.D.		PPL		Experimental	☐
		Protocol		Sham	☐
Date		Pre-op weight			

Sedation			Pre-op		
Drug	Dose	Route	Drug	Dose	Route
Ketamine	15/mg/kg	*S.C.*	Carprofen	4mg/kg	*S.C.*
Domitor	0.25mg/kg	*S.C.*	Antisedan	1.25mg/kg	*I.M.*
			Marcain	1ml (5 x 0.2ml)	2nd to 6th ribs

Post-op Analgesia

Day 0	0.5ml vetergesic immediately post-op			
	A.M.		P.M.	
Day 1	0.05mg/kg buprenorphine + 4mg/kg carprofen	☐	0.05mg/kg buprenorphine	☐
Day 2	0.05mg/kg buprenorphine + 4mg/kg carprofen	☐	0.05mg/kg buprenorphine	☐
Day 3	0.05mg/kg buprenorphine + 4mg/kg carprofen	☐	0.03mg/kg buprenorphine	☐

Weight in kg	Domitor	Ketamine	Antisedan	Carprofen
2.5	0.63	0.38	0.63	0.2
2.6	0.65	0.39	0.65	0.21
2.7	0.68	0.41	0.68	0.22
2.8	0.7	0.42	0.7	0.22
2.9	0.73	0.44	0.73	0.23
3.0	0.75	0.45	0.75	0.24
3.1	0.78	0.47	0.78	0.25
3.2	0.8	0.48	0.8	0.26
3.3	0.83	0.5	0.83	0.26
3.4	0.85	0.51	0.85	0.27
3.5	0.88	0.53	0.88	0.28

Date	Weight	FGS	Appetite	Faeces	Urine	Wound	Temp	Notes

Scoring of 0 - normal, no action required.
Scoring of 1 in any category - moderate - observe and notify NVS if no improvement in 24 hours
Scoring of 2 in any category - severe - NVS notified and animal killed immediately.

Scoring key	Moderate	Severe
Appetite	Reduced	Not eating
Faeces	Frequency, consistency change	Diarrhoea, constipation
Urine	Reduced output	No output
Wound	Inflamed	Obviously infected, open.
Temperature	39.5°C to 41°C	above 41°C or below 38°C

FGS - NC3Rs scoring system.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all studies we will adhere to published current best practice, and local guidelines. All experiments will be carried out, and any data published, in adherence to the ARRIVE guidelines (NC3Rs, Kilkenny et al 2010).

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). We hold regular meetings to plan and review our animal work and information on 3Rs advances will be discussed here.

54. Treatment and monitoring of diabetes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - 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

Diabetes, Wound healing, Insulin, Blood glucose, Medical devices

Animal types	Life stages
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to provide services to evaluate and develop strategies, therapies and devices to monitor and treat diabetes and related illnesses.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 3.9 million people have diabetes (2019 figures) and an estimated further 1 million people are thought to have undiagnosed diabetes, suggesting a total closer to 4.8 million. Prevalance in the UK is rising and is expected to reach 5.3 million by 2025.

Age, family history and ethnicity are risk factors of developing diabetes, whilst obesity is the greatest risk factor, accounting for 80-85% of a person's risk of developing diabetes. A diagnosis of Type 2 diabetes is accompanied by an increased risk of premature death from associated conditions and complications, and ultimately premature death, compared to non-diabetic people. Diabetes related conditions include heart failure (currently estimated to lead to 2000 cases per week in the UK), heart attacks (530 per week), stroke (680 per week) and amputation (169 per week). In the UK, diabetes has led to end stage kidney failure for more than 10000 people, and serious effects on eyesight for more than 1700 people.

Worldwide, currently diabetes is the fifth most common cause of mortality.. An estimated 1.6 million deaths were directly caused by diabetes in 2016. Consequently, diabetes has increasingly become one of the major financial burdens on the UK NHS, accounting for approximately £10 billion every year and 10% of the total NHS budget; this has been projected to account for around 17% of the NHS budget by 2035.

The work conducted in this project can answer lots of questions about new strategies and technologies to monitor and treat diabetes and related illnesses, such as impaired wound healing and eye problems, or complications such as renal, vascular or nerve damage.

Sources: WHO, Diabetes UK,

What outputs do you think you will see at the end of this project?

Data will support product development, product registration, patents, and ultimately lead to new products.

Who or what will benefit from these outputs, and how?

For research Sponsor companies, data can demonstrate proof-of-concept as well as assisting elimination of inadequate candidates prior to further costly development, and further animal research.

In the long-term these studies will benefit patients with diabetes and related conditions. Furthermore the impact of this work may be applicable to multiple diseases, such as wound healing in general.

How will you look to maximise the outputs of this work?

Whilst publication of results would not be discouraged, it is expected that in most cases publication or dissemination will not be possible as the research will be conducted as a commercial service to companies.

Internally, the organisation will review processes and procedures after the completion of experiments, and use that experience to inform and develop future studies.

Species and numbers of animals expected to be used

- Pigs: 180

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

Larger animals (pigs) are useful to evaluate products in the final stages of pre-clinical development. They are often used for testing medical devices due to their physiological similarities to humans: devices and implants of a similar size to those which would be used in humans can be applied; in comparison to smaller mammals (mice and rats) there is a larger blood volume and the response to treatments can be evaluated over a longer period of time.

Typically, what will be done to an animal used in your project?

Animals may experience the following procedures:

Induction of diabetes and manipulation of blood chemistry

Diabetes may be induced in some animals by injection with streptozotocin. Some animals may receive a modified diet, for example low or high fat, or low or high glucose, to induce certain physiological, biochemical or haematological changes. Animals will be habituated for restraint and handling purposes to minimise distress, and pain relief would be provided for any surgical procedures.

Gastrostomy

Under general anaesthesia, a tube would be surgically placed in the stomach and tunneled through the skin to emerge through the skin of the abdomen or back. This is to enable devices to access the stomach and intestines directly. This would be under recovery or terminal anaesthesia. In both cases, pain relief would be provided.

Multiple laparotomies

For devices in the early stages of development, it may be necessary to deliver treatments and observe tissue multiple times within a relatively short time period, e.g. 3 days. In these cases, it may be necessary to perform multiple surgeries to access the stomach ("laparotomy") within a relatively short time period, rather than place a gastrostomy tube.

Endoscopy and cryoablation

Under general anaesthesia, an endoscopic device would be inserted into the stomach or intestines (via a previously created gastrostomy or via the oesophagus). Cryoablation therapy would be delivered to tissues via the endoscopic device. This would be under recovery or terminal anaesthesia. Pain relief would be provided.

Administration of substances and blood sampling

If a drug or cellular treatment is being investigated, these may need to be administered on numerous occasions during the experiment, for example with food or as injections. Substances may be administered to assist with procedures, such as insulin or glucose. We may also need to take multiple blood samples from the animal during the experiment. The animals will need to be restrained for these procedures, and it may be useful to anaesthetise them. To prepare for this the animals are handled regularly to get them used to the staff and reduce any distress caused by restraining them as much as possible. If repeated blood samples are required frequently over a longer period of time, the animal may undergo surgery to place a cannula in a major vessel, which would then be tunnelled under the skin. Pain relief would be provided for any surgical procedures. A "finger-prick" device may be used for taking very small blood samples multiple times during the day. The sites for this would be rotated, and any discomfort would be very brief as this method is very quick.

Rapid manipulation of blood chemistry, e.g. blood glucose levels

Under non-recovery (ie terminal) anaesthesia, animals will undergo invasive surgery to expose major blood vessels from which we can take repeated blood samples and administer substances such as glucose quickly. Pain relief would be provided for the surgical procedure.

General anaesthesia and surgery

Depending on the length of anaesthesia, the recovery can be around 1-2 hours, during which animals will be uncomfortable and disorientated. Where surgery has been performed, pain and discomfort is expected during the subsequent recovery period (days – weeks), which we will manage with pain relief, and antibiotics will be provided if required. These will be administered with food, as injections, or as dressings. Where the animal needs to be anaesthetised repeatedly, for blood sampling or administration of substances for example, these sessions of anaesthesia will be quicker than the initial surgery, but the animal will still need to take medication and need some time to recover afterwards.

What are the expected impacts and/or adverse effects for the animals during your project?

Induction of diabetes

After induction with streptozotocin, a diabetic state may be temporary or permanent, depending on the dose administered and experimental aims. This would affect the regulation of glucose in the blood and may be accompanied by:

- reduced weight gain, weight loss,
- altered appetite,
- thirst and/or dehydration
- increased urination, which may cause discomfort and urinary scalds, or bladder infection, distended abdomen,

- reduced muscle mass, dry, flaky skin,
- impaired thermoregulation,
- impaired immune responses and wound healing which may lead to prolonged infections, effects on eyesight.
- General anaesthesia and surgery

Depending on the length of anaesthesia, recovery can take around 1-2 hours, during which animals will be uncomfortable and disorientated. Where surgery has been performed, pain and discomfort is expected during the subsequent recovery period (days – weeks), which we will manage with pain relief, and antibiotics will be provided if required. These will be administered with food, as injections, or as dressings. Where the animal needs to be anaesthetised repeatedly, for blood sampling or administration of substances for example, these sessions of anaesthesia will be quicker than the initial surgery, but the animal will still need to take medication and need some time to recover afterwards.

Infection

Infection of surgery sites is possible but unlikely as surgery will be performed under aseptic 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)?

All animals are likely to experience moderate severity effects as a result of the protocols described.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

During later stages of product development, it is necessary to evaluate efficacy and safety in a fully functional physiological system. This is also sometimes needed in earlier stages of product development, where physiological dimensions necessitate evaluation in an animal model earlier than usual (for example, demonstrating a new application of a device which is already used in humans, and thus designed for human-sized tissues, vessels or organs). Adequately testing such devices in vitro would not be possible. Using an animal model means technologies can be evaluated in physiologically and clinically relevant conditions, for instance where blood glucose levels can be monitored and manipulated, in

the presence of other simultaneously active physiological and biochemical processes which are difficult to mimic in vitro or in silico.

Which non-animal alternatives did you consider for use in this project?

We expect project proposals to be based on in silico, in vitro or ex vivo data.

Why were they not suitable?

As a provider of in vivo scientific services we discuss supporting information or studies that can be performed in non-animal alternatives or cadavers with individual Sponsors and provide in vivo models when non-animal options have been exhausted.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated animal numbers are based on examples of likely study designs and timespans, for instance: up to two large animal (pigs) studies a year on Protocols 1 and 2, using 15 animals per study (150 animals per 5 years); and up to 6 animals per year on Protocol 3 (30 animals per 5 years).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- Pilot studies with 1-2 animals can be conducted, for example for significant changes to products or procedures, before commencing larger studies - where possible, pilot study data will be included in the main study.
- Pilot studies will be used to validate the procedures in Protocol 1.
- We frequently recommend cadaver studies to Sponsors, and use cadavers for model development and training opportunities.

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 1-2 animals can be conducted, for example for significant changes to products or procedures, or to validate models or methods.
- Pilot studies will be used to validate the procedures in Protocol 1.
- We routinely harvest tissues and organs from experimental animals for research and training purposes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Protocol 1 induces diabetes in pigs. Diabetes will be induced by injecting a chemical, rather than a pancreatectomy: this procedure is a lower risk to the animal. Placing in-dwelling catheters means blood samples can be withdrawn and substances can be administered with minimal restraint and distress to the animal.

Protocol 2 uses pigs for endoscopic cryoablation. For some studies, creating a gastrostomy will require a separate surgical procedure, but will enable the endoscopic device and therapy to be delivered more accurately and easily, rather than having to access the duodenum via the oesophagus and stomach. However, for devices in the early stages of development, it may be necessary to deliver treatments and observe tissue multiple times within a relatively short time period, e.g. 3 days. In these cases, where up to two recovery endoscopic procedures are required within 24 hours of each other, direct access via a laparotomy would be preferable to an extra gastrostomy surgery and longer recovery time. Where more than two recovery endoscopic procedures are required in short spaces of time (e.g. 24hrs between procedures), placement of a gastrostomy would be the preferred choice.

Protocol 3 investigates a non-invasive blood glucose monitor. This protocol takes place under non- recovery general anaesthesia, and pain relief will be provided.

Where surgery is involved in the study, peri- and post-operative care and medication will be provided to reduce pain and minimise the risk of infection.

Why can’t you use animals that are less sentient?

Pigs are often used for these types of experiment because they are a good model for human obesity and diabetes since they have a similar omnivorous diet, a predilection for obesity, a similar cardiovascular anatomy, and a similar metabolism and lipoprotein profile. The ratio of body weight:blood volume is closer to humans than smaller mammals. Their body size, blood volume and growth rates are useful for evaluating techniques and devices over longer treatment/recovery periods (weeks-months) compared to smaller mammals.

The endoscopic device being tested is already used in humans for cryoablation of adenoma in the oesophagus and duodenum. Protocol 2 is investigating a new purpose for the device as a duodenal mucosal resurfacing (DMR) treatment for diabetes. The device is designed for human endoscopy, so would not be suitable to apply to smaller or younger animals. The experiments will be done under recovery anaesthesia to enable evaluation of

haematological and biochemical responses over a longer period of time, during recovery, and histological analysis during longer periods.

For protocol 3, smaller and younger animals (mice, rats, rabbits) would not have a large enough blood volume to produce enough quantitative data, and would be more adversely affected by the magnitude of the changes in blood glucose that would be necessary to adequatley test the device.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We routinely provide post-operative pain relief and antibiotics, and are prepared to extend the provision of these where necessary. All animals are closely monitored immediately after any surgery or intervention, and then observed closely in the days and weeks following, for instance their mood, grooming, movement, weight, eating and drinking, appearance of wounds.

For recovery surgeries, all the animals arrive to the facility at least two weeks before studies start. During this time the animals can get used to the new surroundings and staff, and staff can start to train the animals if this will be needed for the experiment, for instance to stand still for blood sampling. All animals are provided enrichment whilst in the facility: for pigs this would include chains, balls and bars for them to chew.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our facility is GLP-certified and experiments can be performed to GLP-certified standards where required; non-GLP studies are performed to the same standard operating procedures.

The PREPARE guidelines cover many topics that are usually discussed between the research team and Sponsors whilst developing protocols, and an adaptive approach to these will be useful for planning specific studies and continuing the project.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our research staff actively seek opportunities for training and continuing professional development. We circulate the NC3Rs newsletters and take part in IAT and LASA activities. We communicate openly about animal welfare and suggestions and ideas for improvements are welcomed from all staff members.

55. Developing novel tools for kidney disease gene 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

Kidney disease, gene therapy, Podocyte, albuminuria, Glomerular filtration barrier

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 primary aim of the work to be conducted under this licence is to develop new gene therapy vectors to treat common kidney diseases to prevent deaths and other major health care issues associated with 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?

Chronic Kidney Disease (CKD) is a major global public health issue and affects approximately 10% of the worldwide population, with an increasing prevalence with age. Risk factors for CKD include diabetes, hypertension, obesity and metabolic syndrome. The risk of cardiovascular events is increased in patients with CKD, which results in an

estimated 40,000 premature deaths in CKD patients each year in England. This risk increases as CKD progresses. Albuminuria is a feature of CKD and has been shown to be an independent risk factor for progression of CKD as well as an independent risk factor for increased mortality (all-cause and cardiovascular). There are no direct cures for CKD and this work aims to develop novel gene therapy tools to reduce albuminuria and prevent the progression of CKD to End Stage Kidney Disease (ESRD).

What outputs do you think you will see at the end of this project?

The outputs from this project will be the development of novel gene therapy tools to lower albuminuria and delay/halt progession of CKD to End stage renal failure.

Who or what will benefit from these outputs, and how?

Most kidney diseases have a chronic, complex course, punctuated by periods of intensive (and expensive) treatment such as dialysis and transplantation. Renal disease is a major healthcare issue for the United Kingdom and is expensive with 2% of NHS budget being spent on treating this condition. Chronic Kidney disease (CKD) affects 1 in 8 adults in the UK and there is a shortage of specific treatments for most forms of kidney disease. Current treatment options for kidney disease are at best only partially effective and some of the agents used have serious side effects. This project, aims to develop novel gene therapy tools to provide new treatment options for patients with kidney disease.

How will you look to maximise the outputs of this work?

This work involves collaboration with several groups, both national and international, who will provide tools and expertise for this project. Work will be published as soon as possible under signed intellectual property agreements and all data made available if products are taken to clinical trials.

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.

There is an unmet need for medical therapies for all forms of CKD and we want to develop our novel gene therapy tools to treat several of these more common diseases. Currently there are no in vitro models for studying kidney disease which mirror the complexity of the in vivo situation. Therefore, there is no alternative to the use of animals for the studies that need to be undertaken. Where possible we will use in vitro cell models to undertake preliminary studies to validate and ensure the efficacy of our novel tools prior to use in animal models. We will use well established, validated and commercially available mouse models of human disease.

Typically, what will be done to an animal used in your project?

We will use well established, validated and commercially available mouse models of human kidney disease.

The mice develop kidney disease over a period of 4 weeks to 8 months depending on the model. The mice will receive a single tail vein injection of our gene therapy vector and then will be monitored for the development of disease. The well being of the mice will be regularly monitored by measuring the level of protein in their urine and clinical observations using scoring systems targeted to the relevant mouse model.

What are the expected impacts and/or adverse effects for the animals during your project?

All the mouse models used result in renal dysfunction, leading ultimately to kidney failure. Mice will be assessed routinely for general signs of ill health based on a rodent health record score. We will regularly monitor the level of protein in the urine of these animals so that those with renal dysfunction can be identified at a stage before any overt suffering occurs. The monitoring of proteinuria is an important way to minimise harms in all models used.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

With careful monitoring the severity of the kidney disease induced is expected to be moderate in all cases. Monitoring proteinuria is an important way to minimise harms in all models as it will indicate when kidney disease has initiated so that those with renal dysfunction can be identified, regularly monitored using an animal health score and culled before any overt suffering occurs. It is planned that our gene therapy vector will lead to the abrogation of kidney disease in the 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?

The kidney is a dynamic structure responsible for continuously filtering the blood to remove waste productsCurrently, there are no suitable in vitro models for studying kidney development or function and no realistic prospect of developing such a system within the foreseeable future, Consequently, there is no alternative to the use of animals for the studies that need to be undertaken.

Which non-animal alternatives did you consider for use in this project?

In vitro cell modelsare available to carry out all the preliminary work to ensure that our gene therapy tools behave as we expect them to.

However, these do not accurately mirror the complexity of the kidney in vivo and therefore determine the efficacy of these new tools in treating human kidney diseases then animal models are required.

Why were they not suitable?

Where possible we will use in vitro human and mouse cell models to test our novel gene therapy tools, and these do form a major role in the preliminary studies that will be undertaken. However, to fully investigate and establish the efficacy of these novel gene therapy tools, animal models of human disease must be used. Work in cell models will allow us to develop gene therapy tools that are optimal and work efficiently thus allowing us to both replace and indeed reduce the amount of animals we study

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

All the mouse models to be used in this project are very well established. The majority of the mouse models are commercially available apart from two, which will be obtained from collaborators. Small numbers of animals of both these models will be obtained and the progression of the phenotype confirmed prior to expanding the respective colonies. Therefore, the time-course of disease development in all models to be used will be well defined and thus mouse numbers required to test the efficacy of the gene therapy vectors can be estimated with reasonable confidence.

Extensive preliminary data will be acquired using in vitro human and mouse cell models prior to any mouse work taking place to fully verify the vector biology.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This work will build on extensive preliminary work showing the efficacy of gene therapy product in mouse models. The project will use well established and recognised models of kidney disease so the time-course of disease development in each model is well known. All proposed work has been designed together with an animal statistician and reviewed extensively by experts in the kidney field.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All gene therapy tools will be extensively tested and validated in well established in vitro human and mouse cellular systems prior to use in animal models. We will then minimise the number of animals used by following the principles of reduction by careful experimental design and the use of pilot studies and power analysis. After the optimal gene therapy tool has been developed and tested in these in vitro models, we will initially perform pilot studies to ascertain the correct dose of virus required to induce an effect and then go on to a formal hypothesis driven experiment using already available and well researched mouse kidney disease models. The specific principles we will use to reduce numbers will include: 1) Unbiased studies (using randomisation and blinding), 2) Adequate Power (control variability), so the experiments can adequately answer our hypothesis driven questions.

3) Wide range of applicability (factorial design). 4) Designing experiments in consultation with an expert statistician to ensure that the data is readily amenable to statistical analysis. We will also implement intelligent breeding strategies wherever possible by selecting breeding pairs in which there is a maximal chance of the off-spring having the required genotype for the required model. Although this takes time in developing, and a number of breeding rounds, ultimately it results in many less mice being killed due to the wrong genotype being born.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 rodents, as these are the simplest mammals to have kidneys that resemble that of man. We will use well established and characterized mouse models of human kidney disease. Using these well defined models allows us to design the optimal experiments, reducing the number of animals required and reducing pain, suffering and distress as the timeline of disease development in all cases is already known.

Why can’t you use animals that are less sentient?

Currently the are no in vitro models of kidney disease for testing the efficacy of new therapeutic agents. Although a considerable amount of preliminary work will be done in human and mouse cell culture systems, there is no alternative to the use of animals for the final studies that need to be undertaken. We have chosen to use rodents, as these are the simplest mammals to have kidneys that resemble that of man and we will use very well established, characterized and recognised mice models of human kidney disease already readily available.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For each different genotype to be used the natural history of proteinuria and kidney failure is well defined as these are well established and characterized mouse models of human kidney disease. Monitoring proteinuria is an important way to minimise harms in all models as it will indicate when kidney disease has initiated so that those with renal dysfunction can be identified, regularly monitored using an animal health score and culled before any overt suffering occurs ensuring that no animal exceeds the welfare endpoints defined in this PPL.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult any relevant references listed therein. (Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs.)

Animals will be monitored for signs of pain and distress as disease progresses by experienced veterinarians and animal care technicians with significant experience in these species.

Standard Operating Procedures are employed for animal husbandry and procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs and other relevant information is circulated by AWERB as relevant to PPL Holders. Whenever possible we will implement these refinements into our studies.

'Kidney'on a chip models using human and mouse cell lines which have the potential to reduce animal usage are continually being developed and we will keep constantly keep ourselves informed of the latest research in this area. However, although very useful for preliminary experiments these technologies have not reached the stage yet where they can replace the use of animals for the testing of the gene therapy vectors.

56. Molecular mechanisms of haemostasis and thrombosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Haemostasis, Thrombosis, Platelet, Coagulation/anticoagulant pathways, Atherosclerosis

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 furthering our understanding on how blood clots form in blood vessels under normal and diseased conditions (cardiovascular diseases). We want to undertake this work to improve currently available therapies to reduce unwanted blood clot formation and reduce the risk of cardiovascular diseases such as stroke or heart attacks.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The formation of a blood clot is essential to prevent bleeding. However, in some instances it can prevent blood from flowing normally through the circulatory system and arteries or veins can become blocked (thrombosis). In addition, clots can break off and prevent blood circulation in a different location causing a stroke or a heart attack. Currently, there are treatments available to prevent unwanted blood clots such as aspirin or anticoagulants, however they are not 100% efficacious. Indeed, every year 4 million people in Europe still die of cardiovascular diseases (160,000 in the UK alone). Therefore, a better understanding of the vessel wall, cells and molecules that circulate in our blood that are involved in clot formation will enable to identify new ways for treating/preventing thrombosis and fatty build-up in the artery wall, a disease called atherosclerosis.

What outputs do you think you will see at the end of this project?

The ultimate goal of this project is to find new ways to prevent blood clot formation. This will start by gaining new scientific knowledge about how the vessel wall, cells and clotting molecules that circulate in our blood interact in a normal clot to prevent bleeding (a process called haemostasis) and in pathological conditions where excessive clot formation can block arteries and veins (thrombosis). We hope to find new agents to treat thrombosis by increasing anticoagulants naturally present in our blood, by preventing blood cells such

as platelets and white blood cells to interact or by inhibiting important molecules driving clot formation. We have been publishing our findings in academic journals and presenting our work at international conferences and will continue to do so.

Product outputs

- Specific genetically altered mice for gene studies.
- Tissue
- New agents (antibodies) that potentially could be further tested in pre-clinical studies to assess their efficacy in preventing blood clots and ultimately protect against thrombosis and other cardiovascular 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 provide a further understanding of how clot formation occur which should be of interest for research scientist/clinicians in the field but also could have a broader impact as platelets have been involved in various human diseases (e.g. cancer, infections, sepsis). We will also promote and publish any refinements or best practice we identify during this project and make post-mortem tissue available for personal license holders.

In the medium term the pharmaceutical industry will be interested in potential novel therapeutic targets we identify.

The long-term potential benefits of this study are that data generated may have far-reaching implications for the treatment of thrombosis in humans, benefitting both patients and clinicians by contributing to the development of more effective anti-coagulants or anti-thrombotic agents which will ultimately reduce the economic and health burden caused by cardiovascular diseases.

How will you look to maximise the outputs of this work?

Findings and experimental procedures will be made available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings. We will also seek collaborators with appropriate expertise to ensure the work is progressing smoothly. Data obtained from this project will be disseminated via various means (e.g. websites) through the establishment and various charities.

Species and numbers of animals expected to be used

- Mice: 6730

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 ‘gold-standard’ model for studies on thrombosis and blood clotting. Mice also are a good model of human disease and physiology. A high number of genetically modified mutants are available and there is an extensive amount of work that has already been performed and published. The generation of genetically modified mice is sometime necessary for the study of a particular gene for which the function in unknown or there are no commercial tools available to modify its function.

We will use mostly adult animals as they are of appropriate size for the models described in this project license.

Typically, what will be done to an animal used in your project?

Most animals in our project will be genetically modified but not have any other procedures performed - they will only have samples of their organs and tissues taken for study after they have been humanely killed. Animals may be injected with experimental substances usually once but occasionally more often and small quantities of blood withdrawn. Most animals used in experimental procedures will be under anesthesia with no recovery to study blood clot formation.

Some animals will be subjected to a restriction of blood flow in one of their veins and allowed to recover from the surgery. They are expected to survive the procedure. The clot is allowed to form over few days but most animals in this model will be culled after 48h post-surgery. Some animals may be anesthetised for imaging purposes to be able to follow the formation of a clot in each animal.

To study the formation of plaques in blood vessels, animals will be fed a diet rich in lipids as mice are not prone to atherosclerosis (fatty build-up in the artery wall) unlike humans. Some animals may be anesthetized for imaging purposes to be able to follow the formation of plaques in each animal.

In some instances, animals will be treated with anti-coagulant/antibodies against cells to improve the outcome of clot/plaque formation.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the procedures are expected to cause no more than transient discomfort and no lasting harm.

Animals may be subject to surgical procedures. For all surgical procedures, the animal is under general anaesthesia which is sufficiently deep and stable to ensure the animal is insentient throughout. Animals will be carefully monitored during recovery, and kept warm. If signs of strong discomfort are detected (which may happen rarely) this mouse will be immediately removed from the experiment and humanely killed. For one of the procedure, we will mimic the human scenario where people in bed-ridden position, or long-haul flight develop clot in their lower limbs. For this, flow restriction will be applied in one of the large vein of mice and under careful monitored anaesthesia. Animals usually recover quickly, move and eat normally and don't lose weight. To avoid any suffering mice will received strong pain killers before and after the surgery until the end of the experiment.

A good communication between scientists, vets and animal care technicians will be established to provide highest levels of care. At the end of a procedure mice will be humanely killed in accordance with the approved methods of this project license to avoid any excessive suffering, pain 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 procedures are expected to cause no more than transient discomfort and no lasting harm. This mild severity is expected to represent around 90% of the mice. The remaining would be expected to experience a moderate level of severity as defined in some protocols of this project license. At all time, humane endpoints will be applied to comply with the level of severity of the corresponding 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?

Although valuable information can be obtained from a test tube, this do not reflect physiologic conditions and the complexity involved in the formation of a clot in a blood vessel or when fatty build-up develop in the artery wall. Therefore animal models are necessary to better understand blood clots and to prevent and treat conditions associated with excessive clot formation. Indeed, the vessel wall has a complex architecture and interactions between endothelial cells that line our blood vessels and blood cells or tissues as well as the immune system cannot be reproduced artificially. In addition, substances need to be delivered in the blood to mimic the human scenario and to be able to treat these diseases.

Which non-animal alternatives did you consider for use in this project?

To answer our scientific questions and study blood clotting, we perform experiments in test tubes, on cells grown in the laboratory, with blood and tissues taken from healthy people or people with a disease, and use computer modelling and analysis. We will continue to use these approaches to avoid unnecessary use of animals.

In particular, our lab has developed over the years many different models to mimic clot formation using microfluidic technology (small plastic channels that ressemble blood vessels). They are invaluable to understand how clot formation take place and how cells interact with each other. These models are part of our informed decision to pursue or not

animal work. We are currently developing a model of venous valves in the laboratory to mimic the human scenario where clots tend to form in the valves of the large veins of the legs. Human endothelial cells (which line our blood vessels) are grown with constant circulation of fluids in silicone microchannels with an artificial valve in the middle of the channel. These channels are made by 3D printing in collaboration with the engineering department. After few days, human whole blood is passed in the microchannels and cell interactions can be studied.

Wherever we can, we will use these alternatives to replace experiments on animals in order to study clot formation.

Why were they not suitable?

Although very useful these models made in the laboratory still do not faithfully reproduce a blood vessel and how a novel drug would be efficacious in a whole organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated thinking carefully about the number of experiments we will need to do, using our experience of past/present projects, relevant publications in the field for similar procedures, and by performing statistical power calculations using available online tools.

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 maximise the amount of scientific information possible while using the minimal number of mice. Tools such as the NC3R's Experimental Design Assistant tool as well as published guidelines (ARRIVES) will assist in the experimental design of an experiment, and advice from biostatisticians and other experts in our institute Statistical Services Unit will be taken.

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 be mindful to minimise the number of animals that are used in our project. Techniques will always be optimised in view to reduce the number of animals, including the use of non-invasive novel imaging technologies. This will allow to visualise how the disease (e.g. fatty build-up in the artery wall, thrombosis) develop over time without needing to sacrifice the mice and without surgery. Careful experimental plan will ensure that several tests can be performed using blood samples/tissues from the same mice.

Finally, whenever possible sharing data and resources (e.g. animals, tissues, equipment) with researchers and organisations will be applied.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging Refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the ‘gold-standard’ model for studies on normal (haemostasis) and pathological (thrombosis) blood clotting. A high number of genetically-modified mice are available and an extensive amount of work has been performed and published. Mice also are a good model of human disease and physiology.

No adverse effects are expected with most of the models as they are performed under terminal anaesthesia. For all surgical procedures, the animal is under general anaesthesia which is sufficiently deep and stable to ensure the animal is insentient throughout. Suffering is minimised by careful aseptic technique and administration of substances by skilled scientists. For one thrombosis model (DVT model), although these animals are subjected to recovery surgery, they are expected to survive the procedure. To study the build-up of fat in the vessel wall, animals are given a high fat diet.These are very mild models so don't cause any distress or lasting harm and animals will be killed before they experience any adverse effects of the disease.

A good communication between scientists, vets and animal care technicians will be established to provide highest levels of care. Any animals showing sign of distress or pain that cannot be relieved with analgesics will be killed humanely and immediately to keep the animal suffering to a minimum. In all cases, we will use a protocol that causes the least suffering to the animals to achieve our scientific objectives.

Why can’t you use animals that are less sentient?

We will mostly use mice because they are mammals and the least sentient species in which it is possible to manipulate how genes work, to model the diseases we want to study and measure the function of the heart and blood vessels in ways we need to. We can't use less sentient species such as invertebrates (e.g. nematodes/insects) as they have different respiratory, immune and cardiovascular systems than human. We also need to work with adult animals as we need a fully developed cardiovascular system and we need to work with animals that are fully grown from a practical perspective. For 3 out of 5 of the protocols not involved in the generation and maintenance of GA animals described in this project license, animals are terminally anaesthetised. For the other protocols, animals that will require surgery will be given pre- and post analgesics and will be under general

anaesthesia which is sufficiently deep and stable to ensure the animal is insentient throughout.

Recovery surgery and adult animals will be necessary in some cases for the disease process to develop. Increased monitoring by personal licence holders assisted by animal care technicians will be carried out to ensure the best post-operative care for each animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For each model proposed in this project that involves recovery anaesthesia advice will be seek from the vets to use up to date best anaesthesia and analgesic agents. Animals will receive pain relief to treat any apparent discomfort and before any recovery surgery. Surgeons will be trained and assessed appropriately. Increased monitoring of animals post-surgeries as well as rigorous and comprehensive humane endpoints will be applied. Immediate action will be taken to relive suffering if their welfare does not improve after adequate treatment given. Additional refinement measures to enrich the cage environment of animals fed a high fat diet will involve providing additional tunnels and chew sticks to prevent the animals from developing overgrown teeth.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published guidelines issued by LASA & NC3Rs to ensure the experiments proposed in this project license 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?

For all procedures, we will continually monitor the literature for methods of refinement and consider whether the use of animals is necessary to address the experimental question under investigation. When necessary in order to best implement the advances in the 3Rs, advice will be seek to the vets and animal care technicians.

57. Combating tapeworm infection by investigating genes and genomes

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Tapeworms, Neglected tropical disease, Genomics, Helminths, Parasitism

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 and validate the genes and genetic systems that control development in tapeworms

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Tapeworm infection is responsible for a significant part of the global worm burden, negatively affecting ourselves and our livestock. For example, taeniasis, related to global pork production, is responsible for 1/3 of epilepsy cases in Central America and represents one of the most important neglected tropical diseases caused by these pathogens. Our ability to combat these and other parasitic worms has been significantly advanced in through the characterisation of their genomes. These efforts have relied entirely on laboratory model systems maintained by a small number of labs located in different countries, for which the system described here has been one of the principal systems. At

present, it represents the most fully-characterised genome of any helminth, making it an important laboratory isolate for the wider scientific community. These data can now be employed for empirical work in the laboratory aimed at understanding gene function (e.g. by enabling gene and protein characterisation and expression analyses) which is essential for evaluating potential targets for therapeutic intervention.

What outputs do you think you will see at the end of this project?

In the short term the cultures will provide the biological material required for genetic manipulations in the laboratory, that over the mid-term will principally include gene expression and bioinformatic studies aimed understanding the function and interrelationships of their gene products. Results will be published in leading peer-reviewed journals and presented at national and international conferences. Products will also include the development of new genomic resources, such as genome-wide expression data, that will be released to the public upon production as raw data, as well as in curated form through a dedicated webpages These data will have immediate use by the scientific society and will increase the value of this model system in biology. Over the longer term these data will be synthesised into a model describing key developmental pathways controlling growth in tapeworms that, together with the genomic data and empirical results that support it, will be informative for developing novel chemotherapeutics.

Who or what will benefit from these outputs, and how?

The characterisation and understanding of the genes that control development in tapeworms will advance efforts to control and eradicate the agents of significant neglected tropical diseases, potentially benefiting millions of people in endemic parts of the globe. Genomic resources and information derived from the culturing of these organisms have immediate benefit to basic and applied researchers in both academia and industry by providing access to the complete suite of the pathogen's genes and gene products. Functional studies of the genes underlying their development will advance efforts to identify new targets for therapeutic intervention.

How will you look to maximise the outputs of this work?

One of the principle means of maximising the outputs of our research will be to make all genomic data publicly available, both as raw data and in curated form, as described above. Such data can be put to use freely and immediately by other researchers and will be especially valuable for those requiring a representative species with a fully characterised genome. We will also maximise our outputs through collaboration with leading investigators at UK academic and genomics institutions, allowing us to address fundamental questions in stem cell and chromosome biology in addition to questions specific to the pathogen. Through collaborative research, these cultures will help to ameliorate the need for multiple labs to maintain cultures of the same model organisms. Because the system typically underpins 2-3 graduate student research projects/year, in addition to research conducted by post- doctoral researchers and visiting scientists, the cultures also underpin post-graduate training in molecular and bioinformatic techniques and serve as the principal source of biological material for all of our empirical and

bioinformatic investigations. We will also use the system, as we have previously, to educate and inform the public about neglected tropical diseases and about the biology of these organisms.

Species and numbers of animals expected to be used

- Mice: 500
- 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.

Tapeworms are flatworm (i.e. Platyhelminthes) parasites that have complex life cycles involving a minimum of two hosts: an invertebrate host in which they undergo larval metamorphosis, and a vertebrate host in which the larvae develop into strobilated (i.e. segmented), sexually mature forms. Because of these host requirements for the completion of their life cycle, tapeworm parasites of rodents make the most practical model systems in the laboratory, allowing for access to all life stages as well as the generation of new individuals.

Since their initial adoption as laboratory models in the 1960s, species of the genus Hymenolepis have been the most widely employed rodent tapeworms for both teaching and research, with the species H. diminuta and H. microstoma being principal among these. Both species utilise grain beetles (e.g.

Tribolium and Tenebrio spp.) as intermediate hosts; however, Hymenolepis diminuta is a natural parasite of rats, whereas H. microstoma is a natural parasite of mice. The species also differ in other aspects of their biology as well as in their amenability to in vitro culture and other laboratory assays, and hence we will maintain cultures of both species.

Larval stages of both species will be reared in beetles using cultures maintained by the applicant. These will provide access to larval stages for both research and for the infection of rodents in order to produce adult worms (that in turn provide eggs used to infect beetles and thus perpetuate the cycle).

Mice and rats are the natural - and required - hosts of these tapeworm species and tolerate infections exceedingly well, producing no pathological consequences. The species being cultured are a common and normal part of the parasite fauna in wild rodents and beetles around the globe. In addition, no stage in the life cycle of these species poses a threat to human infection, while the requirement for development in an intermediate host precludes contamination of other rodents within the animal facility (i.e. direct transmission from rodent to rodent is not possible as the eggs shed in the rodent faeces are only infective to grain beetles).

Typically, what will be done to an animal used in your project?

Tapeworm larvae will be introduced to the rodent's intestinal tract via oral gavage. They will then be housed and maintained under normal laboratory conditions for up to 6 months, depending on the age and numbers of specimens needed, after which they will be humanely killed using a schedule 1 method and the parasites recovered from the intestine for use in genetic research.

What are the expected impacts and/or adverse effects for the animals during your project?

The rodents are expected to experience mild and temporary discomfort during oral gavage, whereas the establishment of the tapeworm infections is not expected to produce any significant adverse effects (they are a normal part of parasite fauna found in rodents).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild; 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?

Investigating tapeworm genes and development requires specimens that provide the genomic DNA, mRNA and whole specimens with which to undertake genetic manipulations in the laboratory. The parasites cannot be cultured in the laboratory without the use of their natural animal hosts.

Which non-animal alternatives did you consider for use in this project?

No non-animal alternative is available. In vitro culture can support limited growth of adult worms for a short duration, but does not support sexual development required for the completion of their life cycle, and therefore cannot be employed to perpetuate the life cycle.

Why were they not suitable?

Because in vitro culture does not support the generation of new worms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 average annual need for specimens over the past 10 years, primarily for investigations involving the visualisation of gene expression in whole specimens (i.e. in situ hybridisation) which allows for genes to be associated with different tissues, organs or regions of the body. Individual mice typically host ~10 worms, which is roughly the number of specimens required for each gene assay, while additional specimens are required to provide genomic DNA and messenger RNA (i.e. expressed genes) samples. On average, 100 mice/year generates ~1,000 adult tapeworm specimens, enough to support ongoing studies and two graduate student projects; allowing us to examine the spatial expression of roughly 100 genes of interest per year, as well as providing samples used for genomic investigation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We maintain no more than 10 animals at all times for the purposes of ensuring perpetuation of the parasite's life cycles in the laboratory. Additional rodents will be used to generate specimens only when required, for example during student projects. In this way, we only use animals when they are needed and in numbers relating to the specific number of genes of interest being investigated.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

During periods of inactivity in the laboratory (such as our current lockdown situation due to covid 19) we will maintain no more than 10 animals for the purposes of perpetuating the life cycle.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 as they are the natural and required hosts of the parasites. Oral gavage will be used to introduce tapeworm larvae to the rodent's gut as it is considered to

be most effective and least distressing method to introduce the infection. No additional or other procedures is performed.

Why can't you use animals that are less sentient?

All species of tapeworm require both an invertebrate (intermediate) and vertebrate (final) host to complete their life cycle. The tapeworm species Hymenolepis microstoma and H. diminuta are enteric parasites of mice and rats, respectively, and as adults cannot survive in other animals. Beetles host larval stages of the parasites, but do not support adult development. Our main model will be H. microstoma which is hosted by mice which are considered less sentient than rats (and this is reflected in the lower number of rats cf. mice). However, there are differences in the biology of these two species, including their amenability to in vitro culture in the laboratory. Unlike H. microstoma, limited growth in vitro of H. diminuta has been achieved by other labs and thus we will employ this laboratory model specifically for the development of laboratory assays that require in vitro culture.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will closely monitor the health of the animals and immediately terminate the procedure for any individual in apparent distress or poor health.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will perform oral gavage following the Laboratory Animal Science Association guidelines, employing the use of flexible catheter and a total volume not exceeding 100 microliters.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Primarily through information provided by the Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS). The Animals Scientific Procedures Act (ASPA) and the National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs) websites will also be consulted regularly.

58. Efficacy and safety of feed additives / ingredients for farm animal species

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

feed additive, efficacy, safety, farm animals

Animal types	Life stages
Chicken, turkey	adult, juvenile, neonate
Cattle	adult, pregnant, juvenile, aged
Sheep	juvenile, adult, pregnant, aged
Pigs	juvenile, adult, pregnant, aged
Goats	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to generate data from in vivo efficacy and safety studies in the target animal species to be used:

- to develop and market feed additives, products and substances for use in nutritional supplementation, zootechnical performance and aiding gut health.
- in dossiers submitted for the registration of feed additives, products and substances for use in nutritional supplementation, zootechnical performance and aiding gut health.
- to determine the nutritive value and appropriate diet inclusion rates of novel feedstuffs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 protect human health, animal health and the environment, feed additives have to undergo an efficacy and safety assessment. According to the European Parliament and Council Regulation (EC) No 1831/2003, only additives that have been through an authorisation procedure may be placed on the market. EFSA is responsible for conducting the evaluation of the data submitted requesting authorisations. Authorisations are granted for specific animal species, specific age of animal, specific conditions of use and for ten year periods. The EFSA publications "Guidance on the assessment of the safety of feed additives for the target species" (2017) and "Guidance on the assessment of the efficacy of feed additives" (2018), provide guidance on how to conduct and report studies concerning in vivo efficacy and safety (tolerance) studies. Additives are classed into the following categories:

Technological additives (e.g. preservatives, antioxidants, acidity regulators)

Sensory additives (e.g. flavours, colorants)

Nutritional additives (e.g. vitamins, minerals, amino acids, trace elements)

Zootechnical additives (e.g. digestibility enhancers, gut flora stabilisers)

Coccidiostats and histomonostats

In an application for authorisation, the submitted dossier must include copies of the study reports that demonstrate that the feed additive does not have an adverse effect on animal health or the environment and shall favourably affect animal performance or welfare, satisfy the nutritional needs of the target animal and favourably affect the environmental consequences. Studies undertaken on this project will be reported to the sponsor who may submit them to EFSA for evaluation of additive registration.

Enzymes and probiotics, classed as zootechnical additives have been previously studied at this establishment. In the future, work on additives from other classes may be required.

The process leading to a marketing authorisation for a new product is in five stages. The stages are listed below indicating the stages where work at this establishment will occur.

Initial Assessment, including product discovery, validation and assay development and initial screening.

Optimisation and Development, includingrRefinement and tolerance testing - safety studies and ‘look see’ development studies in target species.

Target Species Trials, including efficacy studies in the target species.

Marketing Authorisation, including efficacy studies to confirm application rate.

Marketing Studies, including commercial support studies.

In addition commercial feed companies are looking at novel feedingstuffs to determine their nutritive value, optimum inclusion rate and impact on animal performance, welfare

and the environment. There has been a recent interest in developing by-products of the newly established bio fuel industry, which would otherwise have no other market and would go to waste e.g. distillers dark grains and solubles (DDGS) and yeasts. These products are classed as animal feedingstuffs (Feedingstuff Regulations, 2010) so authorisation for their use through EFSA does not need to be sought.

On occasion there may be a requirement to establish the safety / tolerance levels of feeding novel feedingstuffs and feed additives. For feed additives, EFSA state the tolerance group of animals would normally be offered a feed additive at 10 times the normal maximum dose for the product. At this dose, haematology and blood chemistry need to be assessed from the animals in the tolerance group. EFSA also state that if a 100 times the normal maximum dose is offered to animals and can be shown to be tolerated without adverse effects, then haematology and blood chemistry is not required.

The majority of the work covered by this licence is commercially driven, although some may be government or grant funded. Developing new feed additives to improve animal performance, health and welfare or have a positive environmental impact is a long complex process. Following the identification of candidate products, in vivo toxicity studies are carried out, followed by similar studies using rat models for example, prior to the product being tested in the target animal species.

Numerous successful studies were conducted under the previous licence, this is a continuation of that licence. Most of the studies were regulatory and conducted according to EFSA guidance. These investigated the efficacy and tolerance of feed additives in target animal species. A range of feed additives were investigated, but enzymes were the most common. Studies were mainly conducted using pigs, chickens and turkeys at various stages of development, but studies were also conducted using cattle. Study results will have been used in regulatory dossier submissions, so that the feed additives could be marketed.

What outputs do you think you will see at the end of this project?

The outputs will be the production of reports of studies completed for sponsors which will be acceptable for regulators. Reports will describe the conduct of studies, according to regulatory and guidance documents, and will come to conclusions on the efficacy / safety of feed additives.

In an application for authorisation, the submitted dossier must include copies of the study reports that demonstrate that the feed additive does not have an adverse effect on animal health or the environment and shall favourably affect animal performance or welfare, satisfy the nutritional needs of the target animal and favourably affect the environmental consequences. Studies undertaken on this project will be reported to the sponsor who may submit them to EFSA for evaluation of additive registration.

The introduction of new products to the market is within the control of the sponsors and there is little communication from the commercial sponsor on their plans for products once studies have been reported.

Who or what will benefit from these outputs, and how?

The primary benefit of this project is the provision of safety and efficacy data to facilitate sound regulatory decisions on the use of feed additives in animal feed.

The benefits of work delivered under this licence are to develop feed additives and ingredients that have the potential to improve animal performance, health and welfare and have a positive impact on the environment.

In order to get approval for feed additives it is necessary to generate data of appropriate quality and accuracy in target animal species in response to inclusion of feed additives. These data will be used in the development of a number of safe and effective feed additive products e.g. enzymes, yeasts and probiotics. There is a mandatory requirement to conduct studies to demonstrate the safety and efficacy of feed additive products before they are marketed in the EU. Therefore the accurate testing of such products to ensure they work and are safe to use, protects animal health, human health and the environment.

The project may also generate accurate data on the nutritive value and optimum feeding levels of novel feeding stuffs. New by-products from the biofuel industry will be available to the feed industry in the UK. Research to determine their nutritional value and develop safe feeding levels to maintain animal performance and health will be required.

As animals are increasingly bred for maximum performance, their requirements for the main nutrients (energy and protein quality) as well as vitamins, minerals, trace elements, amino acids will increase to maintain their health and welfare. Much work was undertaken by the Agricultural Research Council in the 1980s to determine animal requirements by production level in each species e.g. cow milk yield or pig daily liveweight gain. Now that production levels have increased, further research is required to understand animal nutrient requirements. This establishment has the facilities, resources and ability to undertake digestibility, balance and performance studies to help the industry better understand the requirements of high genetic merit animal farmed in the UK today.

How will you look to maximise the outputs of this work?

The outputs of the studies are the production of reports which meet the requirements of regulators. Reports are generally commercial confidential to the sponsor and are rarely published / disseminated, other than with the regulator. This establishment can maximise the output by trying to ensure that studies are conducted / reported so that they are acceptable to regulators. This involves ensuring that regulatory guidelines are followed in the conduct and reporting of studies.

Species and numbers of animals expected to be used

- Domestic fowl: 10,000
- Cattle: 100
- Sheep: 100
- Pigs: 1500
- Goats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

Initial assessments of new feed additives are undertaken in vitro to determine their effect on the target nutrient (e.g. phytase enzymes on cereal phytate). Currently, it is not possible to model the response of the whole animal to a feed additive which therefore necessitates the use of animals in studies. Initially, safety of the additive is typically assessed on the basis of toxicological studies performed in vivo, usually on laboratory animals. EFSA states that efficacy and tolerance studies must be undertaken in the individual target animal species to enable authorisation for the use of feed additive products in a specific species.

Food producing species have different digestive systems e.g. ruminant and mono-gastric, and the nutritive value of an ingredient is different between species. Novel feedstuffs are required to be tested in each target species.

The same applies to assessment of novel ingredients, there are no in vitro tests or prediction models to measure the response of an animal to the ingredient or determine optimum feeding levels.

Typically, what will be done to an animal used in your project?

The administration of the test substance. This will usually be incorporated into the feed or water which will be offered either ad libitum or in restricted amounts. Alternatively, it may be administered by capsule or bolus. The duration of administration may range overone or more production stages over the animal's lifetime. EFSA have published guidance on the minimum duration of tolerance and efficacy studies, for example the minimum duration for an efficacy study in weaner / grower pigs is 42 days, for finishing pigs is 70 days and for sows for reproduction is two cycles (from insemination to the end of the second weaning period).

Variation in the composition, constituents, quality and/or availability of the diet and/or drinking water. This may be required in order to assess the ability of a test substance to compensate for a deficiency

i.e. dietary enzymes are claimed to make previously bound elements available to animals.

Exposure to altered environmental conditions (e.g. increased temperature or spiking the bedding with dirty bedding) to cause a stress to the animals and then monitor the effect of the test substance with this management. This is about trying to test efficacy in an environment more akin to a typical farm and not the sanitised environment typical of this establishment. This is an attempt to replicate typical farm conditions so that the efficacy of the feed additive can be tested in a typical farm situation.

Negative control diets may be required as comparators for diets with the test substance added. These diets may cause low feed intake and/or performance.

The use of electronic capsules to capture or transmit data while in transit through the gut could provide valuable additional information on the digestion process and how specific ingredients and additives affect it. This would be a useful non-invasive method of providing additional data with potentially few adverse effects on the animal. An example of such a capsule is the Heidelberg pH capsule which has been in use in human medicine for over 45 years. It is a high frequency micro-electronic transmitter designed specifically for inter-abdominal pH monitoring. The capsules micro-electronics are encapsulated in medical grade acrylic which is hypoallergenic.

Some feed additives (for example probiotics, prebiotics and acids) have been claimed to benefit gut health or to bind with bacteria in vitro, which might benefit both animal health and food safety. The efficacy of such products in helping to reduce the incidence of harmful bacteria might be tested by dosing birds with field isolates of, for example, Salmonella, E coli, Clostridia, etc, and analysing faecal samples collected directly from the animals, from the floor or post mortem.

The data and products collected to determine the effect of dietary treatments, covered on this licence are:

In vivo

Daily observations of animal health and welfare by the technician and scientific team, of all species.

Observation of animal health and welfare by a veterinary surgeon, as described in the study protocol, for safety studies of all species.

Feed intakes will be determined by the difference between feed offered and feed refused. On occasion individual intakes are required to achieve accurate data, which will require animals to be housed individually.

For scientific / research purposes, weight gain will be determined by weighing animals at the start and end of a feeding period. Data will be used to calculate feed conversion ratio.

Blood samples, one off or serial samples from superficial blood vessels, analysed for metabolic profiles and nutrient analysis.

Faeces and urine samples for the analysis of the main nutrients (e.g. dry matter, energy, protein calcium and phosphorus) to calculate nutrient digestibility and retention of dietary treatments. For total collection over three to five days, pigs are housed in metabolic balance pens for up to five weeks and poultry are housed on slatted floors.

Faeces samples collected by swab, faecal loop or finger may be required in order to determine the effect of dietary constituents on the excretion of nutrients, metabolites or other components (i.e. microflora).

What are the expected impacts and/or adverse effects for the animals during your project?

Procedures undertaken enable the collection of biological material e.g. blood, faeces and urine, to determine the effect of the diet on metabolism or digestibility. Procedures are unlikely to cause adverse effects in excess of mild severity. The protocol allows for moderate severity of up to 15% weight loss in animals (except poultry) to enable assessment of tolerance feeding levels. Control measures and determination of endpoints are clearly defined.

In most cases the adverse effects are likely to be minimal or mild. Where adverse effects are anticipated, animals will be monitored regularly to ensure that the moderate severity limits are not exceeded. Where adverse effects are observed we will intervene to ensure that severity limits are not exceeded.

Where at all possible, animals will be returned to farm or sent for commercial slaughter following veterinary assessment that they are fully recovered from the procedure and pose no risk to human health, animal health or the environment. Where this cannot occur (an unregistered product for example), animals will be klled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Under the current Project Licence (PPL PP7973038) the following severities have been assessed to date:
- Animals in each severity by species (%)

	Sub-threshold	Mild	Moderate	Severe
Pig	0	100	0	0
Poultry	13.2	86.8	0	0
All species	13.2	86.8	0	0

- This covers a total of 2480 animals since the project commenced in March 2021 to the end of December 2021. These severities were assessed according to the combination of the regulated procedures conducted and adverse effects observed. It is anticipated that there will be a similar outcome each year of the project.

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?

Initial assessments of new feed additives are undertaken in vitro to determine their effect on the target nutrient (e.g. phytase enzymes on cereal phytate). Currently, it is not possible to model the response of the whole animal to a feed additive which therefore necessitates the use of animals in studies. Initially, safety of the additive is typically assessed on the basis of toxicological studies performed in vivo, usually on laboratory animals. EFSA states that efficacy and tolerance studies must be undertaken in the individual target animal species to enable authorisation for the use of feed additive products in a specific species.

Food producing species have different digestive systems e.g. ruminant and mono-gastric, and the nutritive value of an ingredient is different between species. Novel feedstuffs are required to be tested in each target species.

The same applies to assessment of novel ingredients, there are no in vitro tests or prediction models to measure the response of an animal to the ingredient or determine optimum feeding levels.

Which non-animal alternatives did you consider for use in this project?

None, as the regulatory authorities require target animal species to be used in studies designed to determine safety and efficacy.

Why were they not suitable?

The regulatory authorities require target animal species to be used in studies designed to determine safety and 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 experience with the last Project Licence, which expired in March 2021. This licence is a continuation of the work conducted under that licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The sponsor requests a number of treatment groups containing feed additive(s) at different inclusion rates. The best number of treatment replicates and animals per replicate are determined depending on the type of feed additive and the species and age of the target animal. This aims to ensure that where there are differences in performance e.g. daily liveweight gain, feed conversion ratio, etc. between treatment diets that there are sufficient animals to show a statistical difference at $P<0.05$. Conversely, too many animals must not be used, if having less on study would have shown the same result.

Information has been gathered on statistical design of feed additive studies which shows the different responses depending on animal species, age, product and main performance parameter measured. As much information as possible on expected performance response to treatment is obtained from the sponsor and the number of replicates per treatment is discussed and agreed with a statistician. EFSA guidance gives details on the statistical power, suggesting greater or equal to 80% for pigs and poultry and 75% for ruminants. The statistician uses power of detection, predicted differences to be detected, past information on animal response to a product type, to determine level of replication. All study designs are discussed by the AWERB to ensure the right number of animals is used.

Individual penning, using one animal per replicate is used when appropriate, with all species except growing poultry. When the variability between individuals in the parameter measured is low this gives more robust data and reduces the number of animals used. For example this study design is used for grower pigs at Drayton. Weaner pigs are more variable and vulnerable, so three pigs per replicate are used to get the most robust data and ensure replicate numbers are maintained. Individual poultry have variable performance and are therefore group penned at commercial stocking densities. Group housing is representative of farm practice, which is important as the feed additive / ingredient being tested will be used in commercial production. On study completion, the statistician determines the best type of statistical analysis for each dataset, this is normally analysis of variance with determination of least significant differences.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Screening of animals while on source farms can sometimes help to select the most suitable animals for enrolment onto studies. This can help to reduce the numbers of animals unnecessarily used. This might involve taking samples (blood or faeces for example) or physical examination of candidate animals.

Pilot studies with a small number of animals can help verify / confirm procedures selected before conducting a larger studies involving many more animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The methods employed on studies are usually dictated / implied by the guidelines. This project licence has a wide scope so that it encompasses a wide range of models and methods.

Animals are inspected regularly by animal care staff, and twice each day as a minimum. As a minimum, the Named Animal Care and Welfare Officer inspects all livestock weekly and the Named Veterinary Surgeon inspects all animals monthly. There are methods in place to evaluate adverse reactions, e.g. scoring systems, and these help to ensure that interventions are carried out in order to prevent severity limits being exceeded.

Methods of killing other than those in Schedule 1 are proposed as they are, in our experience, more humane. The methods and limits proposed are confluent with council regulation 1099/2009 on the protection of animals at the time of killing.

Where possible animals will be penned in groups of similar animals. Where individual penning is required, animals will be within sight and sound of compatriots.

Animal husbandry is to a very high standard, by experienced staff following up to date guidelines and regulations. Prompt veterinary attention is provided to ill animals, which are observed closely until resolved.

Why can’t you use animals that are less sentient?

It is a requirement in the guidelines that the test animals used should be representative of the age, sex and class for which the claim is made. Use of species other than the target animal species may be made in exceptional cases, e.g. in the case of minor species, but their use would need to be justified.

How will you refine the procedures you’re using to minimise the welfare costs (harms) for the animals?

When adverse reactions are anticipated and/or observed, the frequency of observation is increased and continuous monitoring will be conducted to help ensure that severity limits are not breached. If unexpected adverse reactions are observed, or the frequency is higher than expected, procedures are in place to ensure that staff with responsibilities under A(SP)A are informed.

Environmental enrichments will be provided where possible and these are tailored to the requirements of the species. Dust baths, perches, nest boxes, toys and auditory stimuli (radio) will be provided for poultry. Toys will be provided for pigs and nesting material or farrowing pigs. Feed for adult pigs may be offered on the bedding, to stimulate natural foraging / rooting behaviour or intact bales of straw may be added to the pens.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Regulation (EC) No 1831/2003 of the European Parliament and of the Council on Additives for Use in Animal Nutrition. www.irmm.jrc.ec.europa.eu

Guidance on the assessment of the safety of feed additives for the target species. EFSA Journal 2017; 15(10): 5021. www.efsa.europa.eu

Guidance on the assessment of the efficacy of feed additives. EFSA Journal 2018:16(5): 5274. www.efsa.europa.eu

The Animal Feed (England) Regulations 2010. No 2503. www.legislation.gov.uk

Defra. Codes of Recommendations for the Welfare: Laying Hens, Meat Chickens and Breeding Chickens, Pigs, Cattle, Sheep, Goats and Turkeys. www.defra.gov.uk/food-farm/animals/welfare

Agricultural Research Council (1980) The Nutrient Requirements of Ruminant Livestock. Agricultural Research Council (1981) The Nutrient Requirements of Pigs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The methods employed on studies are usually dictated / implied by the guidelines. We will monitor for updates to guidance documents and husbandry methods, and will implement any changes with immediate effect.

59. Characterisation of a mouse model of Barth Syndrome

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

heart, tafazzin, genetics, lipid, metabolism

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this study is to characterise and analyse a mouse model of Barth Syndrome, an inherited genetic metabolic disorder caused by a mutation in the gene Tafazzin (Taz).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Barth Syndrome is an inherited genetic condition, which is sometimes fatal. The symptoms of this condition include heart problems, muscle weakness, feeding problems and susceptibility to infection. Analysis of a mouse model of this disease will allow us to understand how a single genetic alteration causes such a wide variety of symptoms. This will allow the development of treatments to reverse the effects of this disorder.

What outputs do you think you will see at the end of this project?

New Information: These studies offer the potential to better understand the processes that go wrong in metabolic disorders and how these cause the clinical problems identified in affected individuals.

Publications: Work arising from these studies will be published in scientific journals and presented at national and international meetings to disseminate knowledge (to scientists and clinicians). We will also publicise our results to the public at open evenings, social media, and on our website.

Products: This work may lead to the identification of drug targets which might be used as a therapeutic for metabolic disease such as Barth Syndrome. We have generated the first model of Barth Syndrome by mimicking the genetic changes observed in patients, and this model has been distributed to many laboratories throughout the world.

Who or what will benefit from these outputs, and how?

This work is aimed directly at benefitting individuals with Barth Syndrome, who will benefit firstly from a better understanding of how this disorder affects them. This should allow a better management of the clinical consequences of condition in affected individuals. The knowledge gained will also be of interest to the scientific community (short term) by expanding knowledge of mitochondrial diseases. By the end of this project this work may allow us to develop new therapeutic approaches (long term) benefiting patients who suffer from metabolic disorders such as Barth Syndrome.

How will you look to maximise the outputs of this work?

We will collaborate with a number of different labs,each with different areas of expertise, to be able to better understand the consequences of the genetic alteration. Our mouse model will be given freely to other researchers in the field to study and develop treatments for the condition. In addition, our work will be published in scientific journals and we will attend relevant scientific meetings to discuss and disseminate our findings with other researchers as well as the affected individuals and their families.

Species and numbers of animals expected to be used

- Mice: 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.

Using genetically altered (GA) mice allows us to assess the relevance of these genetic changes in the normal physiological and disease settings using the complete animal. This is particularly relevant when studying metabolic disorders. We are using GA mice to model how a particular gene alteration associated with a genetic disorder called Barth Syndrome manifests the clinical symptoms in these patients.

Typically, what will be done to an animal used in your project?

Genetically altered animals will be bred together to achieve specific cohorts of interest which will be aged and tissue harvested to determine the effects of these genetic changes in the whole animal. Some of the animals (<20%) may be put on a high fat diet to modify their metabolism and test how these specific genetic changes adapt to these metabolic conditions. A small number of animals (<10%) will undergo ultrasound imaging to help with this analysis. To better understand these changes we may administer labelling reagents prior to humanely culling animals, which aids our analysis of the tissue.

Animals will be maximally aged to 12 months of age.

What are the expected impacts and/or adverse effects for the animals during your project?

Normal animals placed on high fat diet may gain weight but are healthy otherwise. Males with a Taz gene mutation have a slightly reduced body weight but this does not cause welfare concerns and the mice are otherwise healthy.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the animals on this project will have a subthreshold severity meaning they will show no harm. A small proportion (<20%) will be placed on a high fat diet and/or undergo non-invasive imaging which maximally will be a moderate severity due to repeated anaesthesia.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Studying metabolism in the lab has obvious limitations and the true effect on energy metabolism as a result of dietary changes can best be achieved in a living organism. Long-term consequence of altered metabolism (i.e. diabetes) is also best modelled in the living animal. Furthermore, tissues are made up of different cell types which can all contribute to cues influencing the reaction to metabolic stresses which is often difficult to model in non-animal alternatives.

Which non-animal alternatives did you consider for use in this project?

Our mouse experiments are an extension of robust laboratory investigations from work on cultured cells and only progress using mice when sufficient rationale is obtained based on cell culture, including embryonic stem cells and where possible colonies of cells

resembling mouse tissues.

Why were they not suitable?

Metabolic disorders are difficult to model in a dish and for the long term effects these are best carried out in the mouse. In particular mutation of Taz gene has different consequences in, for example, heart cells and liver cells. Of these heart cells are the most relevant to the human disorder, but adult heart cells do not survive well in culture. Aspects of the Taz phenotype also rely on communication between different cell types.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 the design of such experiments and the statistical approaches required to analyse metabolic alterations. We often use inbred strains of mice which reduces experimental variability. Most of the animals will be used in a breeding programme to attain the relevant genetic alterations. Many experimental animals require to carry multiple genetically altered alleles simultaneously, so this involves breeding of multiple strains carrying these different alleles. Where possible excess animals from breeding are shared with projects ongoing in our facility. The estimates also take into account that for example, non-invasive imaging techniques will be carried out to facilitate as much data from a smaller number of animals as possible. Sperm/embryos will be frozen from lines not immediately required for scientific studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Numbers are calculated based on our own experience, using pilot studies in the first instance, to inform on how many mice we require with advice from our in-house statisticians. We try to scale up experiments and stop recruiting into cohorts when we achieve a statistically significant result.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We use inbred strains of mice which are nearly identical to other in genotype resulting in less variability between animals and allow us to use fewer animals to achieve a statistically significant result. We routinely perform pilot experiments using only a few animals, before scaling up to the appropriate numbers for a full study. We maximise the breeding programme to generate the most effective breeders to create the genetics of interest.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using a mouse model with a knockout of the Taz gene. This is identical to the genetic alteration in affected individuals with Barth Syndrome. We will use widely applied techniques to study metabolism, primarily using changes to diet (e.g. high fat diet) which requires less handling of animals. When animals are on a high fat diet, regular monitoring of weight/welfare will allow us to complete studies at the earliest endpoint in which we observe a significant result to prevent unnecessary suffering by extending the study period. Some animals will be imaged to look directly at the metabolic consequences of the genetic alteration of the Taz gene. The imaging is non-invasive and performed using anaesthesia. We are using state-of-the-art imaging equipment which undergoes rigorous testing, quality control procedures, regular maintenance and calibration. Imaging agents are formulated to clinical standards.

Why can’t you use animals that are less sentient?

The mouse is a mammal and warm-blooded which shares many features of human metabolism not found in other cold-blooded species such as flies and worms. Drosophila and zebrafish with a Taz mutation have been studied and provide some information about the role of Taz function, but these do not display the physiological abnormalities observed in cardiac and skeletal muscle. Additionally these models cannot be adequately used and manipulated to study the long range effects of gene alteration in the whole organism and do not allow development and testing of potential therapies. With the ease of manipulating the genetics of the mouse, this makes the mouse the best model organism to understand the genetic changes observed in metabolic diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals are health checked daily in addition to the routine monitoring by the researcher. Where any animal shows abnormal behaviour this animal is placed on enhanced inspection.

Animals on a high fat diet are weighed at regular intervals to detect early clinical signs.

For imaging experiments animals need to be anaesthetised. Body temperature will be maintained during anaesthesia with the use of heated imaging beds. Normal mouse food may be supplemented with gel diet or other treats to encourage eating and drinking after anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The researchers and animal facility staff at the Institute regularly review the NC3Rs website. In addition, we adhere to both the PREPARE guidelines: Planning Research and

Experimental Procedures on Animals: Recommendations for Excellence as well as the 'Animal Research: Reporting In Vivo Experiments (ARRIVE) Guidelines'. These are complementary checklists for researchers designed to improve the planning and reporting of animal research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We continually review our processes and take advice from the Named Veterinary Surgeons, Home Office Inspectors and the NC3Rs website. Our technical staff are very proactive in adopting 3Rs advancements such as non-aversion handling and single-use needles.

60. Genetic alteration and distribution of Xenopus frogs for scientific use

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
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Xenopus, resources, cell and developmental biology, transgenic lines, gene editing

Animal types	Life stages
Xenopus laevis	adult, embryo, juvenile, neonate
Xenopus tropicalis	adult, embryo, juvenile, neonate
Xenopus borealis	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?

Xenopus frogs are used to discover new knowledge about how cells and organisms work, to understand how chemical released into the environment may affect animals and to study human diseases. The aim of this programme is to ensure that many users of the Xenopus models for biomedical and environmental research have straightforward and efficient access to all of the high quality resources that they need.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The African clawed frogs Xenopus laevis and Xenopus tropicalis have a long history of successful use in research; they are used for discovery science in cell and developmental biology, ecotoxicology and regulatory testing. The recent growth in the number of researchers using them is very likely to continue for two main reasons; first, their large cell size and the fact that we know which each cell in the embryo becomes, make Xenopus the organism of choice for analysing all of the molecules that regulate early development at the same time and in the same embryonic cell. Second, the ability to make specific changes to the accurately-sequenced genome very easily and efficiently, particularly in X. tropicalis, means that gene function can be studied successfully in embryos and tadpoles, often without having to breed them. This has opened a new area in Xenopus-based research for the rapid and cost-effective analysis of human genetic diseases. To support this research, resource centres have been established to make, curate and supply the many genetically altered lines the community needs.

What outputs do you think you will see at the end of this project?

At the end of the project we will have current and newly-made genetically altered lines of frogs fully characterised and easily available to researchers across the world. This will result in a more effective research community using Xenopus frogs. As part of the wider programme improvements to frog welfare will be sought continuously and this has been successful in the past, with measures taken and widely adopted to decrease the stress experienced by male frogs due to travel for example.

Who or what will benefit from these outputs, and how?

The outputs from this programme will benefit users of the Xenopus model by making their materials more consistent, providing access to the full range of Xenopus methods (including to users of other experimental models) and allowing groups that use Xenopus beyond stages where the head to tail axis is formed no longer to keep frogs. Together these benefit the frogs themselves by reducing the numbers used and ensuring that good practice is shared in terms of refinement.

The identification and characterisation of gene function will be of benefit to developmental and cell biologists studying basic biology. They will be able to integrate the data about the function of these genes into the genetic regulatory networks that drive development. This will lead to a better understanding of developmental and cellular processes. Now that Xenopus can be manipulated genetically, researchers and clinicians studying human genetic diseases will also benefit from the lines made to support their phenotype analysis or as part of testing whether a specific change to the genome causes a disease.

How will you look to maximise the outputs of this work?

We already work with more than 200 labs across the world, providing resources that underpin their collaborations and publications. We also present our work at all meetings that focus on this model organism and publish methods and original research papers. We also publish collaboratively, for example with labs that do not normally use Xenopus as their main experimental model and with labs that do not keep frogs themselves.

The reach and ease of access by the frog community to existing and new Xenopus lines is greatly enhanced by placing the animals on the community's NIH-funded bioinformatics resource, Xenbase. The lines are not only available as a searchable database but also

linked to the relevant genepage(s), making it almost certain that someone studying a particular gene will become aware of the availability of any relevant transgenic or mutant line.

We also work closely with the US Xenopus resource, exchanging newly-made lines annually to ensure that there is a duplicate of each and to minimise the transatlantic travel of animals, with concomitant increased ease of obtaining the lines for users locally.

Species and numbers of animals expected to be used

- Xenopus laevis: 6350
- Xenopus tropicalis: 11250
- Other amphibians: 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.

Xenopus are used since the entire aim of this programme is to supply Xenopus and related resources to the research community. Adult animals are needed to produce oocytes, sperm, eggs and embryos/tadpoles. Oocytes are used for studies on ion channels and receptor function, sperm are used to propagate dominant, genetically altered lines, eggs are used for biochemical analysis of DNA repair and the control of cell division, embryos/tadpoles are used for studies of cell and developmental biology and to test whether chemicals that might escape to the environment are dangerous. X. laevis remain the most commonly-used species, since they have particularly robust embryos that can be manipulated both physically and by some genetic methods. X. tropicalis have slightly less robust embryos but are diploid and have very few gene duplications compared with other model vertebrates, this makes them particularly suited to genetic studies, including those on human diseases. X. borealis are very seldom used and we keep few of them, however they are used to understand cross-species interactions since they are similar to X. laevis and transplants between them work well.

Typically, what will be done to an animal used in your project?

For adult animals which are used to produce embryos for these experiments the females have an injection of hormone and the next day are gently massaged to stimulate them to lay eggs, since this procedure causes very little discomfort to the animal it can be repeated 15 times at 90 day intervals. The eggs are fertilised using sperm from male frogs that have been killed humanely. One type of genetic manipulation needed to alter genes in frogs requires oocytes, these are removed from the ovary of a frog under anaesthetic. High quality oocytes for these experiments are not found in all frogs, so frogs that produce high quality oocytes will be re-used up to 4 times.

The genetically-altered tadpoles produced from these embryos and oocytes often carry a reporter gene to allow specific cells, sub-cellular organelles or proteins to be seen; these are not expected to be harmful. However some will be loss of function animals that are defective in a specific developmental or cellular regulatory mechanism. In addition to the adverse effect of this, they may be anaesthetised (up to 4 times) to allow them to be

observed.

What are the expected impacts and/or adverse effects for the animals during your project?

For the adult animals that produce eggs for embryos, long experience shows that the adverse impact is very limited. Fewer than 1 in 500 animals shows any sign of egg retention or infection at the injection site. If they have not recovered after 48 hours of treatment these animals are killed humanely, limiting the period of suffering. Occasionally (0 - 5%, depending on unidentified factors) we see deaths due to coelomitis post-ovulation. We are taking steps to reduce these occurrences with some success. For oocyte removal from X. tropicalis the situation is much less clear since it is only being performed in two establishments in the US, direct contact with them reports that this is much the same as when the same operation was done routinely on X. laevis and that infection or failure to heal has not yet occurred in the limited number of animals used. Were either of these to occur then after 48 hours of treatment these animals would be killed humanely, limiting the period of suffering.

For the affected tadpoles there will be diverse adverse effects including: heart or circulation defects, failure of the brain to develop normally producing seizures or altered behaviour, gut defects limiting the ability to feed properly and there may even be alterations in overall body structure. These tadpoles are normally killed humanely after less than three weeks but earlier if there are any clear health problems, endpoints revealed by poor swimming, a lack of feeding or responses to stimulation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Xenopus for gamete production: Mild (30%) Xenopus for oocyte production: Moderate (1%) Genetically altered tadpoles: Mild (69%)

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 project is to produce Xenopus and related resources. Intact animals are needed for the studies in user laboratories since they investigate developmental processes that involve interactions between multiple cell types in the context of an intact organism.

Which non-animal alternatives did you consider for use in this project?

None

Why were they not suitable?

Genetically altered or normal Xenopus adults, eggs, oocytes and embryos cannot be produced without the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is largely based on the use of animals on the preceding licence. For example we are making around 600 sets of X. laevis embryos to send directly to users each year, added to this are the animals used to curate and test the lines held in the centre and to make and assess new lines for users. Large numbers of GA embryos are inevitably made when testing whether mutations/transgenes are in the germ line and often these have to be grown (normally as sets of 50 - 100) beyond the feeding stage for phenotypes to be analysed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The reduction of animals is built into the programme: keeping genetically altered lines at resource centres reduces the number of animals held; using frozen sperm reduces the number of males required to produce a set number of embryos; making and curating lines with dedicated, experienced scientists reduces the number of animals required; sharing oocytes and embryos between multiple laboratories also reduces overall use of Xenopus.
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 a dedicated co-ordinator who ensures that the shipping of resources is optimised to share animal products effectively. Secondly we share eggs and oocytes across the four Xenopus-using research groups based here. A large whiteboard is used to plan each week's experiments to minimise the number of adult 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.

Of the experimental models used in biomedical research, Xenopus frogs provide a good balance between similarity to humans and limited suffering. The accessibility and large numbers of Xenopus offspring lead to limited suffering since few adults are used to produce them and there is no need for surgery to access embryos as in models closer to humans such as mammals. Xenopus are tetrapods and share their genome organisation closely with humans, they have lungs, legs and are generally more closely related in every sense to humans than zebrafish, which is the other vertebrate model animal with similar advantages to frogs in terms of minimisation of suffering.

Why can’t you use animals that are less sentient?

The users of the centre already use oocytes, eggs, embryos or tadpoles for their work thus taking advantage of immature life stages where possible. These are produced by hormonal stimulation of adults. The next least sentient model organism is the zebrafish, which lacks a number of anatomical similarities with tetrapods and has a distinct genomic organisation, thus making it significantly less similar to humans than the frog model. Amphibia have an almost complete suite of mammalian pain receptors and many of the neural circuits needed to transmit and process pain (reviewed below). Even as adults however they lack a part of the brain, the cerebral or limbic cortex, that from brain imaging and lesion experiments in humans produces the negative emotional aspects of pain (reviewed below). This evidence supports the more limited ability of amphibia to respond to pain than mammals; this is even more true of the larval form in which the vast majority of this programme is based.

Analgesia in Amphibians: preclinical studies and clinical applications. C Stevens. Vet Clin North Am Exot Anim Pract. 2011; 14, 33-44.

Cognitive and emotional control of pain and its disruption in chronic pain. M Bushnell et al. Nat Rev Neurosci. 2013; 14, 502-11

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We refine the procedures by: close monitoring of animals after procedures, moving genetically altered animals as sperm/testes rather than as animals and eliminating surgery with recovery from procedures whenever possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The new "frog book" is about to be published by CSH press including chapters on care and best practice methodologies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are at the centre of the worldwide network for the use of Xenopus and meet monthly over video to receive and disseminate improvements in methodologies and welfare, as well as to ensure that unnecessary duplication of experiments is avoided.

Home Office

61. Defining the role of the senescent cells upon the cancer and pre-malignant microenvironment.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Liver, Cancer, Senescence, Immunity, Endothelium

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?

Senescent cells are the earliest stage of cancer. We seek to understand the interaction between senescent cells, the cells that line blood vessels and cells of the immune system within the liver.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Background
The development of cancer occurs when cells of the body accumulate damage to the genetic code that is present in every cell in our bodies. This generally occurs slowly, but can occur more quickly in parts of the body affected by long-term inflammation, such as

liver cirrhosis or inflammatory bowel disease. Having these illnesses leads to damage to the genetic code and activation of cancer-causing genes. At the earliest stages, prior to our current ability to detect cancer, the body has defence mechanisms to suppress these cancer-causing genes and prevent cancer progressing. One such mechanism is called cellular senescence, where cells of the body sense this damage and respond by irreversibly preventing that cell from dividing any further. Senescent cells also signal to other cells around themselves preventing them from reproducing as well. Senescent cells also attract circulating immune cells that kill the senescent cell and the cancer-causing genes that it contains. Therefore, understanding how senescent cells are detected and killed by the immune system is important and could be a way of treating patients to prevent them getting cancer in the first place.

Immune cells, which circulate in our blood protect us from infection and cancer. They enter different parts of our body by sticking to the walls of the blood vessels when they detect a problem, such as inflammation. Within the liver the blood vessels are lined by sinusoidal endothelial cells, which can attract immune cells into the liver to kill bacteria and viruses. We know that immune cells enter the liver when cancer develops, but we do not know how this happens when cells of the liver become senescent. Nor do we understand whether the endothelial cell is important in this process. If we could understand the processes that cause immune cells to enter the liver to kill senescent cells, then we might be able to prevent cancer developing in the first place, through boosting the body's natural defences. Boosting the immune system to fight cancer has already been shown to be effective in other cancers, such as melanoma.

If we can find out whether it is possible to improve the functioning of the immune system and improve the ability to clear the earliest forms of cancer within the liver, we might be able to design treatments that prevent patients in the future from developing cancer.

What outputs do you think you will see at the end of this project?

If successful, this project has a number of significant outputs:

Scientific advancement and publication

The aim of this project is to increase knowledge of the role of the endothelium (cells which line the blood vessels) and immune system (cells which attack infections and cancer) in combating the earliest forms of cancer. By the end of this project we hope to understand how pre-cancerous, senescent cells, signal to the endothelium and the immune system to prevent cancer from developing and how this system fails.

We will aim to promptly publish and disseminate the results in open access, peer-reviewed, high- impact scientific journals and regular presentations of data at high-profile meetings. Some data that is important for working with industry to develop new medicines may not be released straight-away, until intellectual property restrictions are sorted out.

Drug-development and potential collaborations with industry

Through this work, we may identify drug targets to boost the immune system in the clearance of pre- cancerous cells with the ultimate goal of developing new drugs to work in patients. Any potential drugs or drug targets that we discover would be tested in subsequent projects.

Public engagement

We will use well-established routes at our institution to engage with the wider public, explaining why we believe this research is important.

Who or what will benefit from these outputs, and how?

The following groups will likely be beneficiaries of this project:

Other scientific researchers

This project will be directly of benefit to scientific researchers working in the fields of senescence, liver biology and liver cancer. Scientists will be able to access our newly developed knowledge through:

Publication of results in open access, peer-reviewed, high-impact scientific journals and regular presentation of data at national and international meetings. This will promote new scientific work from other groups around the world.

Sharing of our study data through online open access databases. Generation of publicly available data will drive discovery by other groups, but also prevent the need for repetition of expensive scientific studies.

Future patients

Through development of knowledge about how senescent cells are cleared from the liver, we will be able to design novel immunotherapy treatments to prevent the development or progression of cancer.

Society and the economy

If we are successful and find new treatments, these will be developed in partnership with pharmaceutical companies. In addition, if we can develop new treatments for cancer, this will improve people's health-span: their length of healthy life. Of course, if people live into dependent old age this may increase societal costs later.

How will you look to maximise the outputs of this work?

We wholly support making our published findings available through Open Access. We intend to make data generated during this project open to the wider scientific community and the public, via multiple routes:

Communication

New ideas from this research will be shared with scientists through talks at local, national and international scientific conferences; and educational talks for students. We hope that some of our findings will be of general interest and we will work with the public engagement teams at our institution to make the information interesting and digestible for lay readers.

Dissemination

All the results arising from this project will be submitted to peer-reviewed journals and presented at national and international conferences.

Our published data will be deposited in publicly available databases, allowing access to our findings. Useful reagents derived from this study, will be distributed upon request or deposited in publicly accessible repositories, such as Addgene (https://www.addgene.org).

Engagement

We have previously been active in explaining our work to the general public, both at fundraising events such as public tours of our institution, at science fairs, such as our institution's regular Science Festival and talks at local schools.

Development and exploitation

It is possible that potential new treatments will be generated in this project. We will get advice and support from the Technology Transfer Team at our institution to help develop these further.

Species and numbers of animals expected to be used

- Mice: 14080

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our project intends to study how senescent liver cells communicate with the cells lining the blood vessels and white blood cells of the immune system. The use of mice allows us to study several types of cell at the same time, in the development of cancer.

Mice have very similar biology to humans, particularly in their immune system. They have short development and generation times that will allow us to perform our experiments in a timely and efficient manner. Due to the widespread use of mice in cancer research there are a wealth of scientific tools, like antibodies available and drug doses already worked-out for experimental use in mice. To alter genes or pathways in the liver requires targeted genetic manipulation. There are many well known techniques to genetically-modify different cell types at different times in mice. In some cases this can be achieved through injected DNA molecules or viruses, but in the case of modification of the vascular endothelium will require the use of genetically altered mice. We have used all of these approaches before.

Therefore, the use of mice represents the best animal model for our experiments.

Senescence. We will make senescent liver cells through genetic modifications or using viruses. This is an established technique that we have used in mice that are 6-9 weeks old. It has not been demonstrated to work in other animals or in mice at other life stages.

Chronic liver disease. We are interested in understanding human liver disease; mice can

get a similar form of liver disease. We will give the mice a modified high-fat diet or treat the mice with a drug (carbon tetrachloride), leading to the development of chronic liver disease in 6-9 months. Experiments studying liver disease in mice are in widespread use around the world. There are models of chronic liver disease that work faster or earlier in life, but they do not look like human liver disease

Transplantation. To look at the way different cell types communicate with each other, we can perform transplants of different types of cells together into mice that lack an immune system and then study their growth and interaction. The lack on an immune system is crucial to prevent rejection of the transplanted cells. This is a very straightforward process in mice, where animals lacking immune systems are commercially available.

Typically, what will be done to an animal used in your project?

To manipulate the biology of senescence and chronic liver disease requires targeted genetic manipulation. In some cases this can be achieved through injected DNA molecules or viruses, but in the case of modification of the endothelium will require the use of genetically altered animals. We will also use several other techniques:

Liver-specific genetic alterations: For these mice we will alter gene function within different cell types in the liver using combinations of inducing agents, viruses or drugs. These mice will be monitored by clinical signs or intermittent imaging / blood tests over the course of weeks or in a small minority of mice, months. We will not let the mice develop into old age.

Hydrodynamic tail vein injection: This involves the injection of genes in a large volume of saline into the mouse and results in delivery of the genes straight to the liver cells. We will use this technique to induce senescence within the liver cells before following these mice over the course of a month, or in a small minority of mice, months. We will not let the mice develop into old age.

Induction of chronic liver disease: We will induce chronic liver disease by giving these mice a high-fat diet and / or treating them with carbon tetrachloride (a chemical that damages the liver), whilst altering liver gene function using viruses or drugs. We will follow these mice for several months and monitor them by clinical signs, imaging or blood tests. We will not let the mice develop into old age.

Transplantation: These mice will be injected with complex mixtures of cells, either into the skin, spleen or liver. This allows us to study these cells for prolonged periods of time and see how they interact. These mice will be monitored by clinical signs, periodic imaging or blood tests. In mice with skin tumours, we can measure the tumour size directly with calipers. For mice with liver or spleen tumours, we will monitor the tumour using imaging, such as ultrasound. We will not let the mice develop into old age.

What are the expected impacts and/or adverse effects for the animals during your project?

We are proposing a diverse plan of work that includes several procedures that the mice may find stressful. These can be divided into short-term and long-term impacts:

Short-term impacts

Anaesthesia for procedures: Animals may find single or repeated general anaesthesia stressful. We will ensure that the mice are handled sensitively and keep repeated sessions to a minimum. Anaesthesia will last for less than 2 hours; it may be repeated, but not more than twice in a 24 hour period.

Blood taking / injections: This requires handling and injection which may be painful. This will last only a few minutes.

Treatment with drug or inducing agents: This requires handling which may be stressful. Some substances can be given in modified feed. Some substances need to be given by oral gavage which might be stressful. Some substances need to be given by injection which may be painful. Some agents need repeated dosing. We will ensure that we use the fewest number of doses to achieve our intended effect.

Some inducing agents, like tamoxifen, can be painful when given by injection into the abdomen. We will ensure we use the fewest number of doses required. Tamoxifen can cause weight loss in some mice. We will closely monitor this in the mice receiving this treatment.

Hydrodynamic tail-vein injection: Animals have to be placed in a restrainer, before intravenous injection of a large volume of saline. We inject mice whilst they are awake when they may suffer from pain or distress, as lots of scientific data around the world has been gained in this way before. It might be better to perform this in mice under anaesthetic, but we do not know whether this will give the same results or lead to more mice dying. We intend to compare the two methods during this project. After the injection the mice remain lethargic for around 30 minutes, but then recover without problems in most cases. Mice will only undergo this procedure once.

Transplantation:

Under the skin: We will transplant mixtures of cells under the skin of some mice, to understand the effect of pre-cancerous cells upon endothelial cells. This is a quick procedure that involves one or two injections.

Into the liver or spleen: We will transplant mixtures of cells transplanted in the liver or spleen of some mice. We will do this under general anaesthetic to ensure accurate placement and to minimise stress to the mice. Mice will only undergo this once.

Longer-term impacts

Induction of chronic liver disease: To induce chronic liver disease, we will either:

Give the mice a **modified high-fat diet**. The mice will get fat and so may move around less;

with or without repeated **injections of carbon tetrachloride**. This can take up to a year to develop depending on the dietary modification that we use. This involves repeated injections in the abdomen that could be stressful or painful;

We will not let mice reach the point where the mice start developing problems from liver failure.

Repeated investigations: In some mice we will perform drug treatment or imaging

several times over the course of an experiment. There is a tension here between performing more investigations on a small number of mice or fewer investigations on a larger number of mice. In our application we have set limits to the number of treatments or procedures that a single mouse can undergo, to prevent undue suffering.

Liver tumour or transplanted tumour development: Some mice will develop liver tumours in the longer term after tail-vein injection or dietary modification. Mice undergoing transplantation will develop tumours in the skin, liver or spleen. We will closely monitor animals who might develop tumours and ensure that they do not develop signs of distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice	% of total
Mild	54.5%
Moderate	45.5%

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?

When the liver is damaged, cancer of the liver can develop. Our goal is to understand how different cells within the liver interact when the liver is damaged and what goes wrong to allow cancer to develop. Crucially, we need to understand the activation of the immune system in long-term liver damage and whether the endothelium is involved in this process.

To understand the complex relationships between different cells in the liver, we need to study these cells in a living animal, as we cannot study these relationships using cells cultured in a laboratory. Importantly many of these processes are very slow and occur over a period of days or weeks. Animal experiments allow us to perform these experiments over longer time periods.

We can study some of these processes in liver samples obtained from patients, but these only allow us a snapshot. To manipulate or change the behavior of specific cell types in the liver means we need to use genetically-modified animals.

Which non-animal alternatives did you consider for use in this project?

For some experiments we will use cells cultured in the laboratory, that allow us to study how two liver cell types communicate with each other. We have already utilised cultures of endothelial cells with either pre-cancerous senescent cells or cancer cells to determine the

effect upon the endothelial cells.

In addition, we will make use of cutting-edge techniques, such as three dimensional (3D) cultures or liver-derived organoids that more closely resemble the normal liver, where possible. These will allow us to use human liver tissue in the laboratory, something that was not previously possible to do. We can also genetically modify these organoids, replacing some of the mouse experiments.

For experiments where we do not need to manipulate anything, we will study cell behavior in human liver samples removed as part of patients routine clinical care.

Why were they not suitable?

Study of cells grown in the laboratory allow us to develop ideas about which signalling pathways we will then study in living animals. However, these cell experiments do not allow the study of complex interactions, such as the immune system. This can only be studied in living animals:

Cell culture systems can work for two different cell types, but to study more than this is impossible, due to different growth rates and need for different culture conditions.

Cell culture systems cannot keep cells alive for long enough to study the effects of injury over prolonged time scales required to be representative of human chronic liver disease.

Introduction of the complete immune system into cell culture systems is nearly impossible due to the short lived nature of some cells of the immune system and the very slow growth of other white blood cells that protects us from infectious diseases and cancer. To study the effect of induced endothelial behaviours upon the immune system requires a living animal.

Studying human tissues is important to understand human health problems, but we are unable to manipulate specific cell types to see what effect these changes have. Experiments in mice allow us to do this.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animal used in experiments will be carefully predicted based upon data from our previous work and previous published work by other groups. In all cases we will use the minimum number of animals required, for the experiment to give us useful data. We will work with statisticians to make sure that a minimum number of mice are used to generate significant results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whenever we design experiments we are mindful of the 3Rs and the need to continually optimise our plans. We will reduce the number of mice required in our experiments by:

Reducing breeding. We are going to use cutting-edge techniques including specific viruses and genetic-editing technology to improve the efficiency of our breeding programme. These will reduce the number of mice that are bred with the wrong genotype.

Improving experimental design. We will follow the PREPARE guidelines and use experimental planning tools, such as the **NC3R's Experimental Design Assistant** to improve the design of our experiments. We will perform pilot experiments, where we are unsure as to the effect size. This will ensure that we use the minimum number of mice in our experiments. For complex experiments we will seek help from local statisticians to ensure that our experimental designs and mouse numbers are appropriate.

Making sure we get the maximum information from each animal. For each experiment we will try to get the maximum amount of information from each mouse. This will be through repeated scanning, where possible, and analysis of several organs after death. We will also share both samples and data with other scientists, so that experiments do not have to be repeated.

Reporting our findings properly. To report our scientific findings we will follow the ARRIVE guidelines, so that our findings are clearly set-out. This means that the experiments will not need to be repeated by other scientists.

Use of previously generated tissues. Through literature review, we will ensure that similar experiments have not been conducted before. If similar experiments have been conducted we will approach the study authors to ask for access to the banked tissue samples, potentially reducing the number of animals needed to be used in our study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use a number of cutting-edge techniques that will allow a reduction in the numbers of mice that are required to be bred. Complex breeding programmes always generate large numbers of mice of the wrong genotype. Our techniques allow specific genetic modification of liver cells and include:

Hydrodynamic tail-vein (HDTV) injection. We currently use this technique on a regular basis, which allows delivery of small sections of DNA to liver cells, where it is cut and pasted into these cells DNA, altering their behaviour. Using this method we can take any mouse and express any gene that we have designed in the laboratory, within the liver, without the need for complex and wasteful breeding strategies.

Adeno-associated viral (AAV) **injection.** These viruses home to the liver, again allowing gene delivery to liver cells without the need for complex breeding strategies. This strategy is complementary to the HDTV above, but only allows certain genes to be expressed.

In vivo **CrispR-based DNA editing**. This novel technique allows us to use either HDTV or AAV to deliver molecular machines (Cas9 enzymes) allowing us to edit the DNA in liver cells. Therefore, this technique allows us to create specific mutations in the liver cells in a live mouse, without the requirement for complex breeding strategies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 to study the interaction between senescent cells and blood vessel cells in liver disease. To do this we will induce liver cell senescence or chronic liver disease via:

Modifying the DNA of liver cells. Using either hydrodynamic tail-vein (HDTV) injection or injection of adeno-associated viruses (AAVs) will allow us to directly modify the DNA of liver cells, to induce senescence or modify specific genes or pathways. Both are standard techniques that we currently use in our laboratory. The main drawback of the HDTV injection technique is the success rate of delivery; currently about 70% in our hands. Although successfully-injected mice develop shortness of breath and lethargy shortly after the procedure, the mice return to normal within 30 minutes.

The HDTV injection has previously been delivered whilst the mice are awake to allow comparison with previous experiments. A refinement to the technique might be to perform HDTV injection under general anaesthetic, where the mice would experience less pain or distress. We intend to compare the two techniques to ensure that injection under anaesthetic works in the same way.

Induction of chronic liver disease. We will use well established techniques to induce chronic liver disease (CLD) in mice by:

Modifying the diet of the mice to include lots of sugar and fat. These modified diets will lead to chronic liver disease, that looks like human fatty liver disease within 6-9 months;

Treating the mice with a drug called carbon tetrachloride. This leads to liver damage and over time the mice will develop chronic liver disease, that looks like human liver cirrhosis

These techniques are well established and used in many laboratories around the world.

We have significant experience with these models of liver disease, including the problems which may develop. The mice will be closely monitored to ensure they remain in good clinical condition. These interventions will occur in normal or genetically modified mice. To target particular tissues at particularly timepoints we will use conventional transgenic modification:

Genetically modified mice will allow us to study particular genes or pathways, particularly at specific times. Where possible we will use inducible DNA modification (e.g. Cre-LoxP) to specifically target particular tissues and limit the effects on the mouse. This will be particularly important to limit our modifications to the cells that line the blood vessels.

Why can’t you use animals that are less sentient?

Mice develop liver disease, that looks very similar to liver disease in humans, but it occurs much more quickly. Lower species such as flies or worms do not develop chronic liver disease and do not have immune systems like humans. Indeed, their body organs are so different to humans that they are not suitable to study. Mice permit us to study senescence, chronic liver disease and the role of all components of the immune system. Many suitable genetically modified mice already exist and if they don't the tools are available to create them. Therefore, mice represent the most appropriate animal model for our experiments.

We are unable to study earlier stages of development as:

We are interested in senescence or pre-cancer. This has not been demonstrated or produced in embryos or juvenile animals in previous scientific studies.

Induction of senescence or chronic liver disease takes time, which would result in the animals being adults by the time that chronic liver disease had developed.

We are unable to perform experiments under terminal anaesthesia as senescence takes days and chronic liver disease weeks to develop. Performing this under anaesthesia is clearly unfeasible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will seek to constantly improve the experiments that we perform.

Staff development. This work will be performed by experienced doctors, scientists and technicians who will closely monitor the mice. A key refinement is ensuring that staff performing techniques are fully trained. This has been the key in our current facility to improve the success rate of delivery of hydro- dynamic tail-vein injection.

Housing and husbandry. We will ensure that we use environmental enrichment in order to promote normal animal behaviour in our mouse colony. We will plan to house the mice together where possible to avoid loneliness. We will ensure that mice have access to environmental enrichment, such as wooden chew sticks, nest-building materials and fun tunnels, in order to reduce stress. In our current mouse facility we identified that our mouse colony was on a diet too rich in fat and were developing fatty liver disease. On changing the diet to one lower in fat content, the weight of our mice does not increase as much over time and they do not develop fatty liver disease.

Improved experimental design. Pilot experiments will be performed when we try new techniques or models, allowing us to identify any unexpected problems and improve the full experiment. This approach will also mean that we will only use animals that are absolutely necessary.

Improved techniques. We set strict limits to minimise the harm or suffering to the animals used. We will only use a limited number of surgical procedures and ensure that pain is treated after these procedures. We will use general anaesthesia for non-invasive imaging procedures, allowing us improved pictures of the liver. We will monitor the mice throughout the procedure and during the recovery period after anaesthetic. Close monitoring during longer-term experiments will ensure that animal suffering is kept to a

minimum and clinical signs are picked up promptly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have extensive experience in studying senescence and chronic liver disease in mice. We will use this experience during this new project, but will make use of published guidelines to improve our experiments.

We will improve experimental design following the **PREPARE guidelines** and using tools, such as the **NC3Rs experimental design assistant**.

Our animal experimentation programme will be run according to the guidelines and position statements from the **Laboratory animal science association (LASA).** We will care for our mice according to the guidelines laid out in the **Workman et al** (British Journal of Cancer (2010) 102, 1555 – 1577)**,** on animal welfare in cancer research.

During our experiments we will collect data on mouse phenotype and clinical signs according to the
FELASA guidelines.

When reporting our experimental results we will adhere to the **ARRIVE guidelines**, aiming to provide a comprehensive description of experiments and findings.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

During this project I will ensure that I keep abreast of the latest developments in the 3Rs through several sources:

Continuous professional development. I will attend relevant training courses to keep my license current, that will include developments in the 3Rs.

Institutional 3Rs search tool. A web-based search tool that allows scientists to keep up to date with the 3Rs.

Online tools. There are a number of websites for organisations that promote best practice in animal welfare that provide up-to-date information, such as the National Centre for the 3Rs (https://www.nc3rs.org.uk/) or the Norwegian National Consensus platform (https://norecopa.no/).

Peers and colleagues. In biomedical research I have extensive connections with other researchers throughout the UK and the world. I attend numerous conferences, where new findings are presented, allowing improvements in our techniques and experiments. An example of this is the use of AAVs to genetically alter liver cells; I learned this technique from scientific colleagues and have now brought this to our establishment, significantly reducing unwanted breedings.

62. Modelling ciliopathies.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Joubert syndrome, Disease mechanisms, Therapeutic development, Rare disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to better understand the genetic disorders known as ciliopathies (typified by Joubert syndrome) to improve genetic diagnosis by clarifying how the genetic changes affect disease presentation and ultimately to use this knowledge to develop 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 diseases known as ciliopathies affect a range of organs including the brain, eyes and kidneys of children - causing learning difficulties, blindness and life-threatening kidney disease. There are currently no life-sparing treatments beyond dialysis and kidney transplant and the underlying disease mechanisms remain poorly understood.

What outputs do you think you will see at the end of this project?

Outputs will primarily be in the form of publications that will increase understanding of the diseases known as ciliopathies. We have already made significant, high impact,

contributions to the field, eg, demonstrating for the first time the critical importance of previously unrecognised mechanisms affecting the onset and progression of kidney disease.

Who or what will benefit from these outputs, and how?

In the short term, this project will inform the scientific and clinical communities and help to refine and direct research efforts in this area. In the longer term, our studies will benefit patients suffering from these disorders by improving the accuracy of clinical diagnoses and prognoses and it is hoped that the project will progress towards early stage clinical research into novel treatments during the course of this licence.

Furthermore, our work will benefit the wider scientific and clinical communities by serving as a paradigm for how to improve understanding of rare genetic disease using GA mice in parallel with patient cells and other model systems.

How will you look to maximise the outputs of this work?

We publish in high-visibility, internationally-recognised, multidisciplinary research journals that have extensive readership.

We collaborate widely across Europe and USA sharing expertise and technical advice.

We engage extensively with patients, patient groups and charities - locally, nationally and internationally - and regularly publicise our work and our patients in the mainstream media to raise public awareness of rare disease.

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.

We will use juvenile and adult mice carrying genetic alterations analogous to the mutations found in ciliopathy patients in this project. These have been chosen because we will be using the animals to model both the onset and development of the disease and thus need to analyse animals over a range of life stages. In this way we can discover mechanisms that drive both the initiation of disease and influence its progression, and can subsequently investigate therapeutic intervention targeting both early and later stages of the disease.

Typically, what will be done to an animal used in your project?

Typically mice will be bred to produce animals carrying mutations analogous to those found in patients. These mice will either be killed humanely to provide cells and tissues for analysis in laboratory to help identify the underlying mechanisms causing the disease, or will be injected with potential therapeutic agents and their disease monitored, for example

by MRI scanning and urine analysis to assess any therapeutic benefit.

What are the expected impacts and/or adverse effects for the animals during your project?

Typically, genetically altered animals will develop kidney and eye defects that reflect the kidney disease and retinal degeneration typical of the ciliopathies but will show no overt adverse effects. Both conditions progress very slowly in the mouse and, in this regard, mutant mice are indistinguishable from their wild type littermates. However, some animals will present with brain abnormalities that result in a misshapen head caused by fluid on the brain. This is also a progressive condition but can often be ameliorated by delaying weaning of juveniles and providing a soaked dietary supplement - mice that do not respond to interventions will be evident by 5 weeks of age and humanely killed.

Urine analysis (overnight urine collection to assess kidney function) and MRI scanning (up to several hours scanning with anaesthesia to measure kidney structure) may be used to monitor changes in the kidney in live adult mice and these may cause transient stress to the animals whilst they are away from their home 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)?

The severity limit is moderate although the majority (greater than 75%) of mice bred and used on this licence will be mild or sub threshold.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project models developmental disorders that affect multiple organs and the complex interactions that lead to the disease, in particular the genetic components we are interested in, cannot currently be modelled without the use of animals.

Which non-animal alternatives did you consider for use in this project?

Our programme of work is based upon comparing mouse models in parallel with DNA profiles from patients and with cells that we directly isolate from patient urine. In this way the mouse models are directly relevant to the human condition and "standard" (commercially available) cell systems are not useful as their DNA profiles are unknown.

Why were they not suitable?

Standard cell systems used in basic research are not representative of patients - they do not accurately model the DNA changes within the patient, nor do they reflect accurately the organs that are affected in the disease and the complex cellular interactions that underlie these conditions. Furthermore the overwhelming majority of cells used in research laboratories must contain cancerous changes in order to be grown in the laboratory and thus are clearly not suitable for modelling a disease that is not 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?

We have a statistician within our team, who is an expert in population genetics, to help us design our genetic studies and estimate animal numbers needed to generate robust data whilst using the smallest number of animals. Our previous studies within the field indicate that our approach accurately reflects the situation in the patient groups.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We refer to NC3Rs guidance and the Efficient Breeding of Genetically Altered Animals Assessment Framework when designing experiments and routinely conduct power calculations, receiving advice from statisticians to ensure that the minimum number of animals are used in each study to accurately model patient populations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colonies will be monitored carefully to avoid over-production of animals, and animals of specific genotypes that are produced (and tissues) will be shared and used by more than one research group whenever possible. Breeding colonies that are not required in the short/medium term will be stored as frozen embryos or frozen sperm, to minimise continued production of GA animals. Where specific genotypes are readily available from academic or commercial sources, mice will be acquired for each study, to avoid maintaining a breeding colony. We actively engage with a number of national and international initiatives for archiving and sharing of mouse lines and phenotyping information as well as maintaining informal arrangements for sharing lines with collaborators and institutional colleagues.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use genetically altered mice that model developmental syndromes of man and have designed our research to focus on the early stages of these conditions in order to understand the underlying disease mechanisms and identify and test potential treatments whilst minimising the harm to the animals, for example, patients typically present with brain abnormalities, retinal degeneration and can progress to life-limiting complete kidney failure whilst in their teens/early twenties but we have optimised our experimental strategies such that we can identify and test treatments before our mice progress to end stage renal failure, whilst minimising the adverse effects arising from brain and eye abnormalities.

Why can’t you use animals that are less sentient?

We complement our work on mutant mice with zebrafish. Zebrafish can provide important clues as to the fundamental biological processes occurring within cells, however the zebrafish possesses a very primitive kidney (consisting of two nephrons) and has evolved to live in an aquatic environment. In contrast mouse and human kidneys are remarkably similar in terms of structure and function (consisting of thousands of nephrons). Furthermore, the mouse allows treatments to be tested in an animal that has similar circulation/metabolism to that of a human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The majority of genetically altered animals bred under the authority of this project will have no clinically deleterious phenotype, as the lines are maintained as heterozygous animals. Homozygous animals with a deleterious phenotype are produced only when specifically required for the project, or, if produced during a breeding programme, are humanely killed as soon as possible after a welfare problem is identified.

Deleterious effects in some lines develop only after several weeks, and in these instances, breeding programmes are devised to make use of younger animals, that are then humanely killed before the deleterious phenotype is expressed.

During breeding, litter size, number successfully weaned, and any specific adverse effects will be documented by regular (daily) observation of the animals. Husbandry modifications (eg use of soft diet, later weaning dates for smaller juveniles, additional bedding etc) will be adopted as required, as outlined in Wells et al, 2006.

Genotyping will normally be undertaken using tissue removed when ear-notching for identification purposes. When this is not suitable, and tail-biopsy is required, then a specific justification will be submitted to the AWERB before this method is used. If tail biopsy is required, it will be undertaken under brief general anaesthesia (eg with isoflurane) and analgesia (eg meloxicam) administered.

Furthermore, we have refined our use of metabolic cages by introducing "housing" that is

non- absorbent so does not adversely affect the experiment but provides the mouse with an enriched environment within the metabolic cage. We have refined the MRI scanning process such that animals are not transported to and from the MRI facility for each scan but housed adjacent to the MRI scanner to minimise their stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to guidance and training opportunities accessed via AWERB, best practice guidance is regularly sought from websites such as the NC3Rs, Home Office and EU (https://ec.europa.eu/environment/chemicals/lab_animals/index_en.htm). For example, we have recently implemented "non-aversive handling" across our mouse colonies (https://www.nc3rs.org.uk/implementation-non-aversive-mouse-handling-welfare-refinement-and- reduction-mouse-numbers#:~:text=However%2C%20there%20is%20now%20compelling,obtaining%20more%20precise%20research%20results).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of the AWERB mailing list and hold an account with the NC3Rs, receiving the monthly newsletter via email directly from the NC3Rs website. I receive internal institutional updates and alerts highlighting local discussion forums and events on animal welfare that I attend. I also regularly browse the EU guidance for updates on aspects of the 3Rs.

63. Investigating the genetics of cardiovascular disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

hypertension, cardiac hypertophy, genetics, therapy, gene targeting

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?

The purpose of this project is to understand the genetic causes of high blood pressure and its associated impact on the cardiovascular system. Better understanding of these processes and how the genes interact with environmental modifiers such as salt intake or obesity will allow new treatment strategies to be developed for cardiovascular disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

High blood pressure (hypertension) is the leading risk factor for heart disease, kidney disease and stroke, accounting for approximately 12.8% of all annual deaths world-wide. It is estimated that 31.1% of adults (1.39 billion) worldwide had hypertension in 2010, and the prevalence of the disease continues to increase. Hypertension is a complex disease and there are multiple causative factors responsible for its development. Risk factors include age, high sodium intake, obesity, alcohol consumption, physical activity, and

heritability (genetic factors). A range of blood pressure drugs are currently available to treat hypertension. However only one-third of treated patients achieve target levels of blood pressure possibly due to the complexity of the blood pressure control mechanisms. Therefore, there is a need to discover novel pathways and drug targets that will lead to new treatment strategies or enable more effective targeting of current therapies.

What outputs do you think you will see at the end of this project?

Outputs from this project will include increased knowledge in the field of hypertension and its associated cardiovascular diseases as well as the identification of new targets and development of novel treatments.

The results of the project will be presented at scientific conferences and will be written up and published in peer-reviewed scientific journals. The data generated will be used to support future grant applications.

Who or what will benefit from these outputs, and how?

The expected benefits arising from this project are multifold, and will be in relation to both the advancement of scientific knowledge (short-medium term) and further to potential clinical translation of novel therapies (longer term).

Investigations of the candidate genes and proteins identified in our studies will lead to better understanding of the mechanisms responsible for development of hypertension and cardiovascular disease. This novel information will allow us to develop new therapeutic strategies to prevent or reverse the disease processes, which are suitable for clinical application. Improved therapies for hypertension would reduce the number of people suffering and dying from cardiovascular disease and would thereby ease the burden on the healthcare system and the economy.

How will you look to maximise the outputs of this work?

The results from this project will be disseminated to the cardiovascular research scientific community through presentation at national and international scientific conferences, publication in peer reviewed scientific journals and through our local and international collaborative network.

We have already established strong links with medical professionals and pharmaceutical companies, which will assist in the longer term translation of our outputs to the clinic.

Dissemination of data from studies with negative or neutral outcomes will still be possible since publication of neutral and negative studies is now being actively encouraged by some journals.

Species and numbers of animals expected to be used

- Mice: 7000
- Rats: 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 or rats across a range of developmental stages i.e. fetal, neonatal, juvenile, adult and during pregnancy. This is necessary to allow us to understand the underlying mechanisms contributing to cardiovascular disease development at different life stages. Gene expression within a cell is dynamic and can differ depending on the developmental stage of an organism. Different levels of expression can modify the role the gene plays in physiological or pathophysiological processes. Therefore, there is a requirement to assess gene function at different developmental stages.

The rat and mouse models we use are carefully selected based on their unique genetic profiles. Some of the models we use will have established hypertension, which occurs spontaneously (naturally) in adulthood. These models will not only allow us to determine the direct contribution of specific genes on the disease process but will also allow investigation of novel therapeutic strategies which may be applicable for future translation to the clinic. Some of our models (mice and rats) have been genetically modified to inhibit (knock out) the function of specific genes, which allows us to understand the role these genes play in the development of cardiovascular disease.

Typically, what will be done to an animal used in your project?

During this project we will maintain colonies of rat and mouse models of cardiovascular disease, and we will utilise these models to examine the underlying disease mechanisms. We will conduct our investigations in control animals and in animals which have been genetically modified to alter specific genes or proteins that may play a role in the disease process.

We will carry out a range of methods for assessing the characteristics of the cardiovascular system in our rodent models. These methods typically include repeated blood pressure monitoring and repeated echocardiography to examine changes in heart function and structure. Echocardiography is a non- invasive method requiring brief general anaesthesia for imaging purposes. Blood pressure monitoring is typically carried out on a weekly basis by non-invasive means. However, when scientifically relevant, blood pressure can also be monitored continuously and in greater detail using a probe that is surgically implanted under general anaesthesia into the animal. These studies may also involve collection of blood and urine samples at regular intervals. Typically, animals will remain on procedure for 8 weeks.

Some of our studies will also involve modifying the function of the cardiovascular system in our rodent models by altering the diet, or administering drugs or hormones, or altering the function of specific genes in order to modify the disease processes. The majority of these substances are administered to conscious animals, however certain substances requiring continuous delivery, will be administered by minipump, which are surgically implanted subcutaneously under general anaesthesia. These intervention studies will also involve monitoring of blood pressure and non-invasive imaging repeated at regular intervals, as well as the collection of blood and urine samples. Non-invasive monitoring will require brief general anaesthesia. Typically, animals will remain on procedure for up to 12 weeks (with a maximum limit of 16 weeks).

What are the expected impacts and/or adverse effects for the animals during your

project?

Some of the rat models used in this project contain harmful mutations in their genes which result in cardiovascular disease characteristics of moderate severity (i.e. hypertension and stroke). We observe approximately 6% incidence of stroke in male rats. The majority of these strokes are sustained after the age of 4 months, which is the time of established hypertension. Spontaneous strokes are almost never encountered in female hypertensive rats (<1%).

Pain as a result of surgical procedures: all animals undergoing surgery may experience some pain Analgesics will be given in consultation with the NVS and for as long as necessary.

Anaesthetic death (1%)

Weight loss from repeated anaesthesia (1%)

Decreased palatability (1%) and weight loss (1%) as a result of dietary manipulation. Although special diets are not expected to produce any major short-term weight loss or adverse clinical effects in rats or mice (other than the desired cardiovascular modification), palatability issues may result in gradual weight loss over longer intervention periods (e.g. 2 weeks or longer). Body weight will be monitored at regular intervals (typically twice weekly). Body condition scoring will be implemented if palatability issues are anticipated for specific diets.

Acute toxicity (5%) to the maximum viral vector dose in our in-house colony of rats. Sensitivity may be increased in rats from external sources. Acute toxicity is not anticipated in mice. Any animal displaying signs of acute toxicity (e.g., pallor, collapse, tachypnoea) will be immediately culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% of the rats and mice in this project will experience adverse effects that are mild, causing no more than transient discomfort and no lasting harm. Justification:
Although some of the rats contain mutations in genes that can result in cardiovascular disease of moderate severity, the majority of these genetically modified rats (>90%) will be studied before overt clinical signs develop.

The genetically modified mice used in this project show no overt clinical signs.

The adverse effects for the breeding protocol and the majority of the phenotypic characterisation methods are considered mild and transient.

For potentially stressful methods in conscious animals such as tail cuff plethysmography and use of metabolic cages, animals are acclimatised to the equipment prior to measurement and tolerate the procedures well.

44% of the animals in this project will experience adverse effects that are classed as

moderate severity. Justification:

Most of the animals undergoing phenotype assessment and intervention studies will experience multiple monitoring procedures (of mild or moderate severity). In our previous experience of similar studies involving multiple assessment/intervention steps in combination with genetic modifications, we have identified no lasting harm as a result of cumulative adverse effects. Animals on these studies maintain weight, show normal behaviour and do not exceed scoring sheet limits of moderate severity.

A small number of animals may show sensitivity to dietary or pharmacological intervention resulting in body weight loss. These animals are carefully monitored using welfare scoring sheets and are removed from study or killed before exceeding moderate severity limits.

Some animals will experience surgical procedures that are classed as moderate. All animals undergoing these procedures are given analgesia and are allowed time to fully recover from the effects of surgery before undergoing further procedures.

6% of the animals in this project may experience adverse effects that are classed as severe

Some hypertensive rats will experience sudden stroke.

Some mice administered angiotensin II may experience sudden death due to aortic aneurism.

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 nature and complexity of the cardiovascular disease process makes finding alternatives to live animal models extremely difficult. Although cell-based models are available to examine normal and disease mechanisms in cells from blood vessels, the heart and kidneys there are limitations associated with interpretation of results from these models. These cells are mainly derived from juvenile or neonatal animals and therefore results may not be predictive of the cellular response in the intact adult animal. Usually, a single cell type will be grown in isolation (e.g. vascular smooth muscle cells or endothelial cells), whereas in the intact animal, different cell types develop together and function as units. Also, our cell-based models have no blood supply, whereas in the live animal the blood circulation can contribute to disease development by providing a pathway for inflammatory and immune molecules between the target organ and the rest of the body. Therefore, it is important to understand the disease mechanisms in intact organisms, which show similar cardiovascular characteristics to humans.

Which non-animal alternatives did you consider for use in this project?

Where possible we use non-sentient alternatives to live animals. For example, cell culture has replaced the use of animals in some of our functional analysis studies of candidate genes and gene networks and protein/protein interaction studies (e.g. H9C2 cells to determine the role of specific genes in cardiac hypertrophy, and primary cultured mTAL cells to examine the role of specific genes in ion transport mechanisms). Furthermore, we use cell culture to develop and optimise the viral vector gene delivery system in our gene transfer procedures before in-vivo testing is carried out.

To simulate some of the greater complexity of the whole heart, blood vessels or kidney, another consideration is the use of an organ-on-a-chip (OOC), which is a multi-channel 3-D microfluidic cell culture chip that can mimic the functions of human organs. This technology can provide a more detailed and more physiological approach than cell culture studies based on a single cell type. Whilst organ-on- a-chip systems are routinely used for drug efficacy screening, appropriate in-vitro model systems of cardiovascular disease that fully recapitulate the essential changes and mechanisms seen in the disease, are still in the developmental stage.

Why were they not suitable?

Human heart cells are difficult to obtain and can only be maintained in culture for a short period of time and are therefore not suitable as a robust testing model.

Whilst organ-on-a-chip systems are routinely used for drug efficacy screening, appropriate in-vitro model systems of cardiovascular disease that fully recapitulate the essential changes and mechanisms seen in the disease, are still in the developmental stage, and are therefore not currently suitable for use.

Although cell-based models can provide important detailed information on mechanisms occurring within the cell, and will be used alongside our rat and mouse studies, they cannot fully replace the use of live animals for investigation of cardiovascular disease progression where an intact cardiovascular system is necessary.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The total number of rats and mice estimated is based on my experience of performing similar studies over the past 20 years. The numbers will ensure that we would have sufficient animals for breeding purposes and to carry out each protocol enough times to generate data for approximately 20 publications over the duration of the licence. The numbers indicated are the maximum we would use, but the actual numbers are likely to be lower and would depend on the success of current/future grant applications to support the work. The maximum numbers are quoted to ensure it is possible to carry out the studies without the need to repeatedly revise the project licence in future.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Expert statistical advice has been obtained from in-house statisticians and online tools (e.g. NC3R's Experimental Design Assistant) have been utilised during the experimental design phase of this project. For all our studies we follow ARRIVE guidelines to ensure good laboratory practice and transparent scientific reporting.

Use of methods that permit repeated measurements in the same animal allows for optimisation of the number of animals we plan to use. For example, echocardiography permits a range of cardiac parameters to be determined at a number of different time-points, non-invasively in a single animal. The ability to carry out repeated imaging allows the investigation of the evolution of cardiac disease and the potential influence of dietary, or pharmacological or gene transfer interventions to be carried out in a single group of animals removing the need for multiple groups of animals to be humanely killed at each time point. This also increases the statistical power of the studies whereby the effect of an intervention can be assessed in the same animal over time. Also, since data for each time point come from the same group of animals, there is reduced variability in the data sets and consequently smaller group sizes are required when power calculations are carried out for study design.

Where possible, animal numbers will be reduced by using a single positive and/or negative control group when several different treatment groups are being run concurrently. For longitudinal studies, animals may be used as their own controls by carrying out baseline measurements and assessing subsequent change in phenotype in response to treatment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Apart from good experimental design, we will optimise the numbers of animals we use through efficient breeding i.e. striking a balance between the numbers of animals required to assure continued genetic integrity and reduction, replacing breeders before reproductive performance declines, replacing non- productive breeders as soon as possible, using experienced males.

Any tissues harvested when the animals are killed at the end of the study, that are not used for the purposes of this specific project, will be processed and stored for use in future studies by ourselves or will be made available to our collaborators. This bank of stored tissues will reduce the requirement for repeating batches of experimental animals at later date for follow up molecular or histopathological analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The hypertensive rat model used in our studies is an excellent model of human essential hypertension and one of the best existing models of human stroke displaying many characteristics in common with the human disease. Use of this model demonstrates consideration of published guidelines to improve experimental design, in an effort to improve translation from pre-clinical to clinical studies by using a model with many existing co-morbidities. This model has advantages over other hypertensive rat models for use in our studies because it displays salt-sensitivity and end-organ damage (e.g. in kidneys and blood vessels) that other non-stroke prone hypertensive rat strains fail to show.

Genetically modified rat and mouse models (e.g. knock-out, knock-in, conditional) are widely available for detailed functional investigation of specific candidate genes. The most appropriate models will be chosen to assess the candidate genes identified in our human and rodent studies.

Our protocols have been chosen to provide the maximum detailed information necessary for dissecting the complexity of the multiple genetic factors contributing to cardiovascular disease, whilst at the same time ensuring that the animals under investigation experience the least pain, suffering, distress or lasting harm. For example, the radiotelemetry procedure (for direct arterial blood pressure measurement), although requiring an initial surgical procedure will, after complete recovery, allow the continuous monitoring of blood pressure without the stress of repeated handling. Radiotelemetry also provides superior data output allowing assessment of day time and night time variation in blood pressure which not possible with other methods of blood pressure measurement.

Administration of substances will be carried out by the least severe/painful method available. For example, substances may be administered in the diet or in the drinking water rather than by injection. The substances we propose to use for our gene transfer studies are well characterised, and the maximal dose for our rat and mouse colonies has already been established. We will ensure that this maximum dose is never exceeded, and all batches of adenoviruses will be titered using our established in-house techniques.

Animals will always be housed in pairs or groups unless individual housing is required due to welfare reasons or for scientific purposes.

For all studies involving animals we follow ARRIVE guidelines to ensure good laboratory practice and transparent scientific reporting (e.g. we randomise our animals when including them in our experimental studies and our researchers remain ‘blinded’ to the treatment groups until after the study is completed. This reduces unconscious bias in experiments).

Why can’t you use animals that are less sentient?

Mice are the least sentient vertebrate group in which the process of hypertension development and its associated target organ damage in the intact animal can be interrogated. However, the rat is the more tractable model of the human cardiovascular system, displaying greater similarity with humans in terms of disease pathophysiology. The project cannot be entirely conducted in terminally anaesthetised animals because the disease processes develop over a period of weeks.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All of our animals are monitored on a daily basis. Those animals undergoing surgery or other interventions to modify the cardiovascular system will be scored according to a numerical scoring sheet for pain/discomfort/distress in mice & rats, and if necessary will be humanely culled.

Regular non-invasive monitoring by blood pressure measurement or echocardiography will typically identify harmful changes in the cardiovascular system prior to the development of overt clinical signs.

To reduce stress, animals are regularly handled, and will be acclimatised for short periods in metabolic cages and familiarised with the blood pressure recording procedure prior to the actual measurement phase of the study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best practice local guidelines as well as guidance published by the NC3Rs and the Laboratory Animal Science Association (LASA). In addition, we will adhere to ARRIVE guidelines for appropriate transparent reporting of our research outputs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Project Licence holder and members of the research team will ensure continued professional development in the 3Rs area through regular attendance at meetings/workshops such the Animals in Science Regulation Unit (ASRU) annual meeting, 3Rs workshops/ symposiums held at local or national Research Institutes, and also attendance at local training workshops organised by the NTCO.

Home Office

64. Sensory function in zebrafish

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, Deafness, Brain, Sensory systems, Hair cells

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this programme of work is to define the critical physiological steps required for the maturation and function of the auditory (hearing) and vestibular (balance) systems, and how they integrate to other sensory inputs (e.g. visual information) to inform animal behaviour. How biological systems orchestrate these mechanisms are major challenges in the quest to understand human biology and diseases.

Like mammals, zebrafish have auditory and vestibular organs that contain the sensory receptor hair cells. Moreover, the basic biological aspects covered in this proposal are common to all vertebrates. The advantage of using the zebrafish is that we can provide an understanding on how these sensory organs work in vivo, which is currently an unachievable task in mammals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

The sensory systems are key to our daily life. One of such system is the auditory system, which allows us to perceive sound in all of its different forms, from noises to speech and music. The sensitivity of these sense is remarkable, allowing us for example, to detect sounds as low as the drop of a pin or as loud as an explosion or an airplane taking off. It is also key for survival in many animal species, as its speed in processing information is unparalleled among sensory systems. This is the reason why time- critical sports such as sprint races start with a gunshot and not, for example, with a flash of light.

Despite the importance of this sensory system, we still have a poor understanding on how it achieves these unparalleled performances. How does the brain develop its ability to extract information from sounds? How does it combine this information with other sensory modalities to make decisions to drive behaviour? These are critical questions in our quest to understand how the brain works, as well as to understand the physiological basis of sensory disorders.

The research listed in the proposed work is aimed at elucidating how the auditory and vestibular systems are refined in their complexity during development. More importantly, the project aims to determine how auditory and vestibular inputs are processed and integrated with other sensory modalities in the brain, and why genetic and environmental effects lead to deafness.

What outputs do you think you will see at the end of this project?

The main outputs of the proposed project are publications in peer-reviewed journals and the identification of potential therapeutic targets to slow down or even prevent hearing loss and balance disorders. In addition, we use our research to deliver outreach activities in public events and school visits.

Who or what will benefit from these outputs, and how?

Hearing loss is the most common sensory deficit and one of the most prevalent chronic disease in the elderly. About 12 million people in the UK and ~500 million worldwide have disabling hearing loss (>900 million people expected by 2050) [WHO 2021; RNID]. Hearing loss excludes people from basic day-to-day communication, which is associated with significant psychological and medical morbidity, including social isolation and depression. Hearing loss in mid-life is the largest modifiable risk factor for dementia. The exact prevalence of vestibular dysfunction among people is less clear, but it can be as high as 35% in adults aged 40 years or older. The number is largely increasing in the elderly, affecting their quality of life.

Currently, our ability to develop new effective treatments for auditory and vestibular disorders is that very little is known about the underlying molecular and cellular mechanisms leading to their dysfunction.

Since the basic biological aspects involving these sensory systems are common to all vertebrates, we have selected the zebrafish for the proposed program of work because it will allow us to study the molecular and cellular mechanisms in in vivo, which is currently not possible in mammals.

This will offer a great social benefit since it would contribute, in the long term, developing therapeutic intervention to ameliorate or prevent different forms of hearing loss and

deafness, and possibly vestibular dysfunctions.

How will you look to maximise the outputs of this work?

The work will deliver several scientific publications of the mechanisms used by the auditory and vestibular system to develop and function. Dissemination of the data will also be maximised via invited seminars and scientific conferences around the world. The findings will also be disseminated to the general public via the several outreach activities performed by the hearing research group.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 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 use of animals is essential for this project since at present it is the only means of obtaining any fundamental information about the physiological properties of cells in the intact neuronal circuits responsible for sensory perception.

The zebrafish is currently the least sentient animal model to study sensory integration and the mechanisms leading to hearing and vestibular function and dysfunction in vivo. This is not only dictated by the several similarities of these sensory systems among vertebrates, at both molecular and cellular level, but also due to the relatively small size of the zebrafish and the ability to generate zebrafish with similar sensory defects as those present in humans. As such, the finding obtained from using the zebrafish will be used to further our understanding of sensory function and dysfunction in humans.

Because we are interested in studying how sensory systems become specialized and function, we will use zebrafish at all stages of development spanning from egg fertilization up to adult stages. Zebrafish during early stages of development are transparent, allowing us to image deeper into the fish under in vivo conditions, which is not possible with other vertebrates showing comparable sensory systems to that of mammals. From this work we will be able to learn more about how organs, tissues and cells develop. We will look at older stages of fish to be able to understand how these sensory systems function when reaching a mature stage. This allows us to investigate the mechanisms used by the hair cells to become such highly specialized sensory receptors.

This knowledge will then be implemented in future studies in mammals to test for possible common mechanisms, which will help understanding and addressing sensory-related dysfunctions.

Typically, what will be done to an animal used in your project?

The large majority of zebrafish will be immersed into an anaesthetic (non-recovery anaesthesia). Anaesthetized zebrafish will be placed on a chamber in order to perform imaging or electrophysiological measurements from the sensory systems or brain. Adult

zebrafish, which normally require gill movement for breathing will be intubated under anaesthesia (procedure: terminal anaesthesia). Experiments normally last no more than 4 hours.

In a subset of zebrafish, when we have evidence that the anaesthetic is going to alter the normal physiological responses, the fish will be restrained in agarose prior performing any measurements. Restrained zebrafish will be placed on a chamber and cells and neurons from the sensory systems or brain imaged. Experiments normally last no more than 4 hours.

Sometimes, when we need to test the effect of a compound (e.g. the ototoxic aminoglycoside antibiotics), zebrafish will be anaesthetised prior the administration of any compound, these experiments usually last for no more than 4 hours. Zebrafish are recovered and analysed for the effect of the compound at a later timepoint by 1) fixation of the tissue (under non- recovery anaesthesia); 2) anaesthetized or embedded in agarose as described above. The overall procedure can last several days (free swimming fish when not anaesthetized or restrained in agarose).

The use of neuromuscular blocker will be required for a very small number of experiments in larval zebrafish (up to 10 days post-fertilization), when we have evidence that the anaesthetic is compromising our measurements, and the method of restraining is not suitable for the type of procedure (e.g. activating the lateral line of the zebrafish with water motion to simulate swimming behaviour).
Zebrafish will be placed on a recording changer and cells and neurons from the sensory systems imaged. Experiments normally last no more than 2 hours and at the end the animals are killed.

What are the expected impacts and/or adverse effects for the animals during your project?

For all experiments, including those involving the use of the neuromuscular blockers, the adverse effects might include some discomfort and a potential increased stress level. Very rarely, larval and juvenile zebrafish may become unable to swim, although at this age they breathe through the skin. Nevertheless, these potential adverse effects will be monitored during procedures (e.g. ability to swim and heartbeat) and minimized using the best practise. Zebrafish in distress such as inability to swim upright if not linked to vestibular mutations or conditions that differs from siblings (e.g. lethargy or hyperactivity) will be immediately killed by a schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of fish should experience no more than mild severity or non-recovery (typically 90%) with a subset of animals experiencing no more than moderate severity (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?

Understanding the basic biology and diseases in living organisms is a major challenge for scientists. However, complex biological systems that span from the periphery to the central nervous system cannot be replicated in non-living animals (at least at the moment). This is additionally complicated by the fact that the sensory system, such as the auditory and vestibular, are deciphering and integrating several environmental signals. As such, scientists are dealing with a twofold experimental design: how to represent with high fidelity such intricate sensory systems and how to combine this with the large variability and dynamic range of environmental information. Currently, we do not have the answers for all these problems, which is why we need to use living organisms to gain the required information to develop more bench-based and theoretical approaches.

Which non-animal alternatives did you consider for use in this project?

Theoretical models represent an additional approach that can be used to address aspects of our research questions. Currently, we are developing projects that will use this modelling approach to predict animal behaviour. However, models are only as accurate as the information used to inform these models. As such, a meaningful model can only be delivered if we use real measurable data from the biological process under investigation. Currently, this can only be obtained from in vivo animal
experiments. Therefore, theoretical models and in vivo animal experiments must be used in conjunction, at least for now.

Organotypic cultures have been used for some experimental work on mammals, including testing the susceptibility of the hair cells to several ototoxic compounds. However, we still rely on in vivo animal experiments to fully understand organ development and function. These types of cultures have not been developed yet for the zebrafish because of the difficulty associated in explanting the required tissue from the animal.

Why were they not suitable?

Organotypic cultures are not suitable because they lack the complex architecture of the nervous system, which prevents signal processing and sensory integration. Theoretical methods are not suitable because they can only be used to provide ideas on how a system may work, but they cannot address the problem directly. Although animal models are still the only means to understand the intricate mechanisms implicated in the development and synaptic transmission in the nervous system, we are currently working with colleagues to build a computational model of sensory function using the data collected by our experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot

studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used is estimated based on previous work done in our lab for more than 10 years under two previous licenses. Moreover, we use the expertise present in the zebrafish facility at Sheffield, which has helped us to keep the use of zebrafish to a minimum. For complex electrophysiological and imaging functional studies, we will require at least 6 zebrafish per experiments to reach a meaningful statistical analysis. The total number vary a lot depending on how many experiments. In the last two studies, which we have just concluded, we used a few hundred zebrafish.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Several of the approaches used in this proposal (e.g. zebrafish intubation; agarose), and selection of the most appropriate anaesthetic for the different procedures, were developed in my group over the last several years. This has allowed us to drastically reduce the number of zebrafish used under this project.

To reduce animal surplus, we follow the very strict breeding guidelines from our zebrafish facility

In order to decide the sample size needed for the experiments, we use the statistical inference technique suggested in the NC3Rs website.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We follow the Zebrafish Embryo Genotyper (ZEG), which helps to reduce the numbers fish raised by only raising those that are of the correct genotype and required for experiments. We will also use pilot studies where applicable to determine baseline parameters.

All personnel involved in the project undergo a compulsory training in all procedures used prior working with animals, which is designed to keep the number of animals required to a minimum.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The use of the zebrafish as a model demonstrates the use of a species of a lower neurological sensitivity compared with mammals. The large majority of the procedures are designed to test physiological aspects of the sensory systems using imaging and

electrophysiology methods, which are non-invasive techniques and does not require any surgical operation.

Why can’t you use animals that are less sentient?

We need to use animals in which the sensory systems have some strong resemblance to those present in mammals since we want to be able to translate our finding in the future to human; the long-term aim is to devise therapeutic intervention to ameliorate or prevent sensory disorders. There are not alternative animals that can be used for this project that have less sentient, while retaining the same genetic tractability and similarities with the mammalian system. Although most of the procedures will be performed under terminal anaesthesia, some are not possible because of its well- known side effects on the nervous system, including the sensory pathways.

We will use zebrafish from early stages of development up to adult stages because the project is aimed at understanding not only how the sensory systems are put together during development, but also how they function in the fully mature animal. This requires us to investigate zebrafish over a wide range of ages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During our procedures we will carefully monitor the condition of the animals and will apply early endpoints should any notable pain, suffering or distress become apparent. In addition, we are constantly monitoring our experimental endpoints to determine if the questions posed can be answered through reducing experimental time.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To the best of our knowledge there are no published best practice guidelines for the zebrafish work listed in this project license. However, we use philosophies of experimental design advocated by the likes of Festing and Wurbel in order to refine our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will consult our NC3Rs regional programme manager to discuss advances in the field of 3Rs and keep abreast of new 3Rs initiatives through the NC3Rs website and/or attend future 3Rs workshops related to the use of non-mammalian species.

65. Safety, efficacy and immunogenicity of regenerative cellular 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

Regenerative medicine, Stem cells, Cellular therapy, Immune response, Tissue engineering

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant, embryo
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to study the biological behaviour of regenerative cellular therapies when transplanted into rodent models. The purpose is to confirm that the cellular therapies survive after transplantation, are safe, function, and to understand how they are recognised and affected by the immune system. Our priority is to work on development of cellular therapies for liver disease, diabetes, brain disorders (such as Parkinson's Disease), bowel disorders and blood 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?
Many patients with end-stage organ failure die of their disease or need organ transplantation to survive. In cases where treatment is available, such as insulin therapy

for diabetes or dialysis for renal failure, current treatments do not cure the disease and the quality and duration of the patients' lives are severely affected. Cellular therapies, such as those derived from stem cells, represent a promising opportunity to develop methods to repair or replace diseased organs and tissues. The proposed work is essential to ensure that cellular therapies under development for human patients are safe and effective.

What outputs do you think you will see at the end of this project?

The proposed project is expected to lead to new discoveries about the function of regenerative cellular therapies, and how they are recognised and rejected by the immune system. More specifically, we expect to generate at least one cellular therapy and advance it to a stage that it can be investigated further in large animal models. In the long-term (5-7 years), we expect that the findings of this study will result in the design of at least 1 human clinical trial to test the safety and efficacy of a cellular therapy developed in this project. The findings of the studies will be disseminated widely through publications and presentations.

Who or what will benefit from these outputs, and how?

In the short-term (1-3 years), the primary beneficiaries of the proposed project will be other researchers who are also developing cellular therapies for treatment of medical conditions. We anticipate that the findings of this study will be of broad relevance to the research community in this field. In the medium term (3-5 years), we anticipate that industrial companies involved in the manufacture of cellular therapies will also benefit from the findings of this study. These companies and manufacturers are essential for the production of cellular therapies that are eventually used for treatment of patients. In the long-term (5-7 years), this project will benefit patients with conditions such as diabetes, liver disease, bowl disease, blood disorders or Parkinson's disease. The benefit will initially be limited to those patients enrolled in clinical trials investigating the cellular therapies developed in this project. We hope that ultimately (7-10 years) large numbers of patients will benefit from the findings of this study, after the cellular therapies have been shown to be safe and effective in clinical trials and can be manufactured at large scale.

How will you look to maximise the outputs of this work?

We will disseminate all findings of our studies, including unsuccessful approaches, through publication in peer-reviewed journals, presentation at scientific conferences, and through meetings with other researchers. All publications will be open access, including through platforms such as F1000Research. This project includes collaborations with a large number of researchers with expertise in complementary areas. This network will be utilised to maximise the dissemination of the new knowledge gained through this project.

Species and numbers of animals expected to be used

- Mice: 4675
- 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.

Mainly mice will be used in the proposed project. A small number of rats which may be used if the technical aspects of the surgery mean that it cannot be performed safely and effectively on mice due to their small size. It is anticipated that less than 5% of the animals used will be rats and that more than 90% of the mice used will be adults. Mice are the lowest species with a comparable physiology that enables useful information to be gained relating to the safety and effectiveness of human cellular therapies. Importantly, genetic strains of mice are available that allow studies to be designed that can generate valuable information about specific cellular therapies. In a small number of experiments (estimated at less than 5% of total animals used), new-born mice must be used in order to allow for studies investigating how the immune system rejects human cellular therapies. The new-born mice will be approximately 4 days old. Due to their young age, these mice will develop a more functional immune system, which will enable more informative data to be generated in a limited number of specific experiments.

Typically, what will be done to an animal used in your project?

In a typical experiment, the animal will undergo one surgical procedure during which it will be transplanted with a cellular therapy (for example, into the abdomen). The typical animal will then be kept alive for 4-12 weeks before being culled, so that the tissues can be harvested and examined. The animal may undergo blood sampling typically 2-4 times per month during this period. Approximately 50% of the animals will also receive an injection to give them human immune cells (such as white blood cells) to study rejection of cellular therapies. Approximately 25% of the animals may also undergo a procedure such an injection of toxin or oral dosing of a drug into the stomach via a tube. The purpose here will be to induce a disease, such as liver failure or diabetes, to test the effectiveness of the cellular therapies. Approximately 10% of animals may undergo injection of a toxin or drug into the brain, followed by behavioural testing. The typical animal will undergo one surgical procedures and be kept for approximately 12 weeks. The typical animal will then be culled electively while still well and without clinical signs. In some cases, the animals will be culled by removal of organs while under deep general anaesthetic. Newborn mice will only receive one injection of cells to administer immune cells. All all procedures on these mice will be performed later when they are adults.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that most (more than 90%) of animals will recover rapidly and well from stem cell transplantation.

Injections with immune cells (such as white blood cells) is also generally well tolerated. Most (more than 90%) animals will not experience adverse effects from this procedure.

Animals that undergo a procedure to induce a disease, such as diabetes or liver disease, would be expected to display the effects of that disease (such as abnormal glucose levels in diabetes or abnormal liver function in liver disease). In most cases, this would only manifest in the form of abnormal blood results (for example, high glucose levels or abnormal liver tests) and the animals will not display any clinical signs. Injection of toxins into the brain followed by drug testing may results in transient abnormal behaviour such as circling. Circling behaviour is when the animal walks in a circle after administration of the drug. This behaviour is expected to last no more than a few minutes. The circling

behaviour ends when the effects of the drug wear off and is not lead to lasting harm.

In some cases, the animals may experience weight loss, reduced food intake, reduce movement or an abnormal coat. In such cases, the animals will be culled if these clinical signs do not respond to treatment (such as high energy and easily digestible diet) and persist for up to 24 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals that undergo a surgical procedure will be expected to experience moderate severity. This is expected to be the case for the majority (65%) of experimental animals. A small proportion of animals (25%) will only undergo injections and will experience mild severity. The remainder (10%) may be culled for tissue and will experience subthreshold 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 aim of the project is to study the safety and function of cellular therapies and how they are recognised by the immune system. Robust and reliable investigation of these characteristics of the cellular therapies requires the use of animal models that can mimic the biology and diseases encountered in humans. We routinely use extensive laboratory experiments to characterise the cellular therapies without the use of animals. However, complex responses such as their ability to reverse clinical signs of a disease, or rejection by the immune system, cannot be studied comprehensively without animal models. Importantly, data generated from animal models are essential for gaining regulatory permission for the ultimate use of these cellular therapies in human clinical trials.

Which non-animal alternatives did you consider for use in this project?

The majority (more than 90%) of the cellular therapies used in this project will be derived from humans. Mouse cellular therapies may be used as controls in less than 10% of experiments. Importantly, this is itself an significant part of our Replacement strategy. Very few animals will be used to generate cellular therapies: human tissue will be used instead. Moreover, we have and will continue to make extensive use of specialised human cell culture systems, where human cells can be examined for their function in the laboratory. By growing cells in the presence of human immune cells, we are also generating important data about the therapeutic potential of, and the immune response to, our human cellular therapies. Similarly, we utilise human organs, supplied with human blood on specialised

machines, as a site for transplantation of some cellular therapies. This approach further reduces the need for animal experimentation.

Why were they not suitable?

As outlined above, we make extensive use of human tissue and human cells, as well as sophisticated human cell culture systems and human organs examined on machines, to replace the use of animals in many studies. However, these alternatives cannot entirely replace the use of animals as they do not fully replicate the complete repertoire of the biology of the cellular therapies when administered to patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals have been estimated based on the range of studies that are planned, as well as based on the previous similar studies we performed during the last five years. Based on our previous experience, we are able to predict, for each study, the number of animals that are required to generate reliable and reproducible data. Using our previous experience, we are also able to predict how many studies we can perform in a given time period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have generated a wealth of data from previous experiments that enable us to estimate the anticipated effect size and variation in the experimental data. This data will be used to ensure appropriate experimental group sizes. We routinely randomise animals to experimental and treatment groups. All experiments are conducted and/or data analysed in a blinded manner to reduce bias. When a new cellular therapy is under investigation, we will first perform pilot experiments with small animal groups (typically 2-3 animal per group). The pilot experiments will be used to confirm the appropriateness of the experimental design and to generate pilot data to enable group sizes to be formally calculated. We will also use tools such as the NC3Rs Experimental Design Assistant to ensure experiments are appropriately planned to generate reliable and reproducible data. We will also take into consideration any regulatory requirements relating to the reproducibility of the data, in order to ensure data generated from this study is suitable for informing design of future clinical trials.

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 reduce the number of animals we use through a number of simultaneous strategies:

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.

Where possible, we will use each animal as its own control. For example, we will transplant two cellular therapies in two separate sites (for example, into each kidney) in the same animal in the same operation. This will reduce inter-animal variation and reduce the number of animals used.

By generating large quantities of human cells, where appropriate, we can continue to perform new studies using the same human cells. This reduces the variation associated with different human donors and reduce the number of animals used.

By monitoring animals for prolonged durations, and through the use of non-invasive monitoring techniques (such as imaging), we can generate longitudinal data about cellular therapies without the need to cull animals at numerous 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 experimental models and techniques used in this project can be broadly divided into three groups:
Transplantation of cellular therapies into animals to assess the survival and safety of the cellular therapies

Reconstitution of the animals with a human immune system to assess immune response to the cellular therapies

Generation of a disease state in animals to assess ability (function) of cellular therapies in reversing disease

All experimental models have been refined to ensure they cause the least pain and suffering. The disease models have been designed so that biochemical abnormalities (e.g., high blood glucose levels or abnormal liver function tests) can be used as experimental endpoints in the vast majority (more than 95%) of experiments, without animals displaying harmful clinical signs. Importantly, none of the procedures are expected to result in severe clinical signs (such as persistent abnormal behaviour or persistent weight loss). Animals will be culled if they display clinical signs that do not respond to treatment (such as easily digestible food or pain relief medication). Animals therefore will not be permitted to experience lasting harm.

Why can’t you use animals that are less sentient?

Mice are the least sentient animals that can be used to generate valuable data to investigate the therapeutic potential of human cellular therapies. As the response to, and

the function of, cellular therapies takes hours to days to manifest, experiments cannot be performed exclusively under terminal anaesthesia. Rats will be used in a very small number of experiments (approximately 5%), because their larger size will enable some surgical procedures to be performed more successfully and without adverse effects.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In close collaboration with the staff at our animal facility, we have a robust mechanism for the post- operative monitoring of our experimental animals. Animals are also given routine post-operative pain relief medication, which has been proven to be effective in previous similar studies. When adverse effects may be expected, we readily increase the frequency of monitoring to identify animals that may be experiencing adverse effects. We also have our own dedicated animal technicians who ensure that our animals receive close attention if there are any concerns. We have achieved a number of significant refinements during the previous series of studies, including: enhanced environmental enrichment (such as extra card-board housing); use of high-energy or tasty diets to prevent weight loss; improved techniques for transplantation of cells in the kidney or abdomen (such as using special needles to shorten the duration of the procedure) and administration of toxins into a specific organ to prevent spread of the toxin in the whole body. Where adverse effects may be expected, we will perform particularly close and frequent monitoring of animals. These will include the use of observation sheets and body weight records. We will continue to strive to develop new refinements.

A important aim of many of the proposed experiments is to determine how long cellular therapies survive after transplantation. Where possible, we will use imaging methods to monitor the survival of the transplanted cells. Such imaging can be performed using a short anasethetic (lasting less than 5 minutes) and without any harm or surgical procedure on the animal. Importantly, this means that we can obtain important data from the same animals over several weeks, without having to cull animals at multiple time points.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be conducted and reported in adherence to best practice guidelines including those published by the Laboratory Animal Science Association (LASA). We will follow guidelines on record keeping, performing surgery, education and training, and reporting of experimental results. We will also follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines on experimental conduct including study design, randomisation, 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?

As a current project license holder, I am closely involved with the activities of the 3Rs committee at my institution, including the development of recommendations and dissemination of information relating to advances in 3Rs. I intend to continue with my activities, including through review of relevant publications, guidelines and best-practice information.

66. Non-Clinical Safety

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

Toxicity, Rodent, Safety, Assessment

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 characterise the toxicity of test substances and/or monitor concentrations of test substances in the body to enable selection of the best candidates for future development as potential new medicines, help improve drug discovery and/or assist with choosing the correct doses for subsequent studies.
- To develop or improve methods used in our studies to understand toxicity of test substances

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 assist in the selection and development of new medicines that can be used to treat medical conditions in humans and therefore improve

the quality of life for patients. Effective treatments for many diseases are not available because the underlying causes are unknown, and whilst some medicines do relieve symptoms they do not change the underlying causes.

The Company is a science-led healthcare company and our medicines and vaccines are improving the quality of life for patients around the world. However there are still millions of people who would benefit from future advances in medicine development and new or better treatments.

Achievement of the objectives of this project will provide high quality data to help to ensure that only the most favourable substances (medicines) are selected for further development and ultimately reduce the impact of human diseases.

What outputs do you think you will see at the end of this project?

High quality information generated from animal studies conducted under this project is expected to enable decisions to be made on whether a potential new medicine (substance) should continue into the next phase of development or be stopped from development because of unfavourable characteristics.

The information may also be used to improve the way in which potential new medicines are discovered and synthesised by the Company.

The benefit of stopping development of unsuitable substances early in their development is that it will avoid toxicity evaluation in larger studies using more animals, and allow resource to be focussed on other substances, improving the potential of bringing medicines more quickly to patients. In addition, data generated from studies conducted under this project may be used to help choose doses for subsequent studies, help to identify measurements which should be monitored during subsequent toxicology studies or may be used to modify the substance to increase the chance of it successfully becoming a new medicine. The data generated may also provide information that shows how a potential new medicine may cause toxicity which will support assessing the risk for patients who may ultimately take the medicine.

Without these studies progression of potential new medicines to early human studies and to patients cannot occur.

The design of studies conducted under this project will be kept under review and it is possible that further development, validation or refinement may be required (for example due to advances in technology) to improve the information that they provide.

Who or what will benefit from these outputs, and how?

The purpose of this project is to provide data to contribute to the overall process of medicine development with the ultimate goal of developing safe and effective medicines for the benefit of patients. The data generated from small early studies in rodents will be provided to the Company’s Project Development Teams.

The data may be used for:

- Making decisions on which potential medicine candidates show promising characteristics for future development and which medicine candidates show

unfavourable characteristics for which development will be stopped.
- Provision of information to help choose doses that may be used in future studies.
- Helping drug discovery teams choose better candidates for potential medicines.
- Understand how toxicity may have occurred and how findings in animals may also be seen in humans.
- The preliminary toxicity of test substances will be assessed as follows: Assessment of general toxicity
- The purpose of general toxicity studies is to generate data to understand the general effects that a test substance will have in the body usually by assessing effects on various tissues and blood constituents. The goal of these studies is to investigate the relationship between the various doses of the test substance administered and any adverse effects observed.
- Assessment of genetic toxicity

The purpose of genetic toxicity studies is to investigate the test substance's ability to damage genes (i.e. DNA). If the damage is not repaired effectively then this can change the DNA sequence to cause mutations, which result in changes to or loss of gene function. The most serious mutations in cells of the body can lead to cancer or inherited birth defects. Therefore, it is important to identify this potential hazard early in drug development to avoid exposure to humans.

Information may also be generated that can be used within the Company to develop or improve the methods used in our studies to understand toxicity and improve animal welfare. Examples of these may include new ways to measure toxicity (e.g. to detect damage to components of cells), use of new equipment to collect measurements and samples (e.g. use of a specialised camera to measure body temperature or specialised bedding to collect urine), use of new methods (e.g. to enable blood samples with test substance-induced mutations to be stored for longer periods without deterioration).

How will you look to maximise the outputs of this work?

Work conducted under this licence will not be included in scientific publications when it is confidential and is subject to intellectual property constraints. Where these restrictions do not apply, information may be shared (for example work that may involve scientific collaboration with academic or industrial laboratories and be included in scientific publications). Information will also be shared with the wider scientific community if there are potential 3Rs benefits (e.g. refining methods that reduce the impact on animal welfare).

Information generated using this licence is stored in searchable company databases, so that it can be used by all company researchers. The information generated will remain available, even after likely project and personnel changes, and will be a valuable future resource for understanding previous toxicity findings to support company programs for new medicines.

Species and numbers of animals expected to be used

- Mice: 1000
- 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.

For Regulatory Authorities to give permission to conduct trials of potential new medicines in humans they require their safety to be assessed in regulatory studies in animals, generally in two species of mammals according to published guidance. Studies conducted under this project are small preliminary (non- regulatory) studies conducted early in the development process for decision making purposes.
Since they are designed to provide early characterisation of toxicity and may support subsequent regulatory studies it is necessary to conduct them in the same rodent species (rats and mice) intended for use during the subsequent regulatory development programme. The same animal models and methods are used in the preliminary toxicity studies to correlate the findings with the bigger regulatory studies.

Rats and mice will be used for this project because they are acceptable to Regulatory Authorities for the assessment of toxicity. Adult animals will be used since they have developed physiology and organ systems representative of human patients, and align with the ages of animals used in regulatory studies.

Typically, what will be done to an animal used in your project?

During preliminary toxicity studies animals will normally be given a dose of test or control substance once or twice a day via the mouth, nose or injection into a vein, under the skin, into muscle or the abdominal cavity. Usually only one route will be used but rarely, a combination of two of these routes of administration may be used on each dosing occasion (for example where the intended route of administration in humans will not result in high enough blood and/or tissue levels to explore toxicity in animals). Where possible, the same route as that intended for humans will be used. Dosing will typically be carried out for 3 to 14 days but may range from a single dose to 28 days dosing. Typically, three groups of animals will be given different doses of the test substance.

Animals will be restrained to facilitate some dosing routes (e.g. by placing them in clear plastic restraint tubes) typically for under 5 minutes (e,g. when dosing rapidly into a vein) but for up to 4 hours (e.g. for doses breathed in via the nose or when it is necessary to introduce the dose into the vein slowly over a period of time). Prior to dosing by the intravenous route animals may be placed in a warm environment (sometimes with less freedom to move around) at up to 40ºC for up to 15 minutes to assist with locating the appropriate vein.

Animals may be trained for some procedure so that they become used to them before they are used on a study (e.g. prior to dosing, animals may be placed in restraint tubes to get them used to the procedure that will be used during the study).

Typically, animals are expected to experience mild, transient pain and no lasting harm from the procedures used to administer substances.

Blood samples will typically be collected following the first and last dose (and rarely on other occasions) from conscious animals over 24 hour periods during which up to 8 samples will be taken (called a profile) to measure levels of the test substance in the blood

and sometimes to assess any effects that the test substance may have on the components of the blood (e.g blood cells and other elements that normally make up the blood) over the profile. Additional blood samples may be collected on further occasions if a substance is designed to remain in the body for a long time or it is necessary to assess components of the blood in conscious animals on different occasions from when profiles are measured.

Blood may be collected from veins in the tail, legs or neck following introduction of a needle into the vein or by making a small puncture wound over the vein.

Animals will be restrained to assist with blood sampling using clear plastic restraint tubes for typically less than 5 minutes and prior to this they may be placed in a warm environment sometimes with less freedom to move around at up to 40ºC for up to 15 minutes to assist with locating the appropriate vein and dilate the vein to increase blood flow. Conscious animals are expected to experience no more than mild and transient discomfort from blood sampling.

The amount of blood that can be collected from the animals will be kept within carefully controlled limits to prevent adverse effects and only the minimum amount necessary to conduct the measurements will be taken.

In order to assess any effects that the test substance may have caused on the components of the blood, blood samples will also typically be collected at the end of studies with the animals under general anaesthesia. The animals will be killed whilst still anaesthetised. The animals will be aware of the anaesthetic being administered and may experience mild distress but are anticipated to not experience pain.

Other measurements may be necessary to understand the characteristics of the test substance such as:

Collection and analysis of urine that may require animals to be housed singly without food for a period of up to approximately 16 hours to ensure the best quality urine sample. Assessment of effects on the eyes which may require applying drops to the eyes to dilate the pupils, shining a light into the eye to assess pupil constriction or to measure the pressure within the eye by direct contact of test equipment with the eye surface.

An assessment of effects on body temperature either from a microchip injected under the skin or from a temperature probe inserted into the rectum.

These additional procedures are expected to cause no more than mild and transient discomfort.

At the end of the study, the animals will be humanely killed. Tissues may be collected after death and may be weighed and examined to find out if the test substance has caused any changes. Various tissues and /or blood may be used for other purposes (e.g. to determine levels of test substance in them).

Some studies may be performed to develop or improve methods used in our studies to understand toxicity. Dosing and measurements on these studies will be determined by the specific study objectives, but they are expected to not cause more distress or pain than those included on toxicity studies.

What are the expected impacts and/or adverse effects for the animals during your

project?

The most common adverse effects will be loss of body weight or reduced weight gain, and reduction in the amount of food the animals are eating. Other common adverse effects include altered coat condition, reduced activity, postural changes, partially closed eyes, abnormal breathing and fur staining and 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. Generally adverse effects will be absent prior to the next dose or on the next day although some animals may still show slight effects

The majority of animals will experience mild adverse effects with effects of moderate severity estimated to occur in approximately 10% of animals.

In rare cases the severity of adverse effects may worsen rapidly as doses are increased and in these circumstances the animals may experience severe effects or be found dead after dosing despite careful dose selection and close and frequent monitoring.

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.

Expected severity categories and the 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 90% of animals are expected to experience mild adverse effects, approximately 10% will experience moderate adverse effects and less than 1% will experience severe adverse effects.

Approximately 10% of the total number of studies conducted under this project are anticipated to be preliminary genetic toxicity 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?

Regulatory Authorities require the safety of potential new medicines to be evaluated in mammalian species before they will give permission to conduct trials in humans. There are currently no reliable and robust animal alternatives acceptable to Regulatory Authorities for this purpose. Information generated under this project which is used to make decisions to support the development of new medicines therefore needs to be obtained from studies in the same animal species that are intended to be used in subsequent regulatory safety studies.

Which non-animal alternatives did you consider for use in this project?

Whilst there has been much progress with the development of non-animal alternatives such as organoid cultures (three dimensional cellular structures held within a matrix gel), 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 various 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.

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.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 to support the Company’s plans to develop new medicines has been estimated based on experience of similar work and projects previously supported in the Company.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of groups and number of animals in each group are based on core study designs that have been used extensively in studies conducted by the Company. Using the information generated in preliminary studies, the Company has a track record of successfully designing follow on studies and stopping development of unsuitable substances. The numbers of animals used in studies are typical of those used throughout industry.

Where possible blood samples will be collected using microsampling technology. This allows measurements to be made using reduced volumes of blood and for some studies will support a reduction in animal numbers.

A large pool of scientists are available to provide expertise to assist with the design, conduct and interpretation of the studies and all studies undergo internal scientific and ethical review to ensure the best possible designs.

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.

Where appropriate, samples of tissues or body fluids will be collected for use in future investigations. The results from preliminary cell based assays in mammalian cells to assess genetic toxicity are used to decide what measurements are required in subsequent studies evaluating genetic toxicity in animals. Where possible these cell based assays will be planned so that subsequent measurements in animals can be combined into the same study in order to minimise the number of studies and reduce animal use.

For test substances causing damage to genetic material in cell based assays, methods have recently been developed to evaluate them in rodents used in repeat dose toxicity studies. Previously it was necessary to use specially bred animals for this purpose but where possible this assessment can now be conducted in the same animals used for the preliminary toxicity studies and therefore potentially 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?

The animal models used in this project are well established with large historical control databases, and have a proven track record for provision of data aiding assessment of toxicity. Where appropriate, these animal models will be modified and refined, to maximise the toxicity information generated during the study (for example using tissue or blood samples for measurements that would normally be conducted in separate studies).

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.

Where possible microsampling technology will be used to reduce the volume of blood required to assess levels of the test substance in the blood and if feasible the same animals will be used to provide samples to measure levels of test substance in the blood or tissues as those used to assess toxicity.
This allows direct comparison of treatment effects and test substance levels and reduces the number of animals required overall. Where appropriate, samples of tissues or body fluids will be taken for use in future investigations and in some cases, this may avoid the need for separate investigative studies.

Studies may involve ‘staggered starts’ to limit the overall number of adverse effects by reviewing data from previously treated groups of animals before choosing doses for later groups.

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.

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 in order 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.

Studies conducted under this project are small preliminary (non regulatory) rodent studies conducted early in the development process typically to characterise the toxicity of test substances with the potential to become human medicines.

Information from preliminary studies is used to make decisions which include selection of potential new medicines with favourable characteristics for progression into the next phase of development, selection of doses for subsequent studies, helping drug discovery teams choose better candidates and potentially may also help understand the causes of toxicity and whether findings in animals are also seen in humans. They are not designed to provide data to allow Regulatory Authorities to authorise testing of potential new medicines in humans because they do not comply with the study design guidelines required by Regulatory Authorities for this purpose. Such regulatory studies are conducted outside of the Company at Contract Research Organisations. However, the same animal models and methods are used in the preliminary toxicity studies to inform and influence the designs of the bigger regulatory studies.

Preliminary toxicity studies (both general toxicity and genetic toxicity) are generally short studies (typically 3-14 days) using small numbers of animals (with controls as appropriate) to understand how effects relate to the dose of test substance administered. The first phase of genetic toxicity studies is used to determine a maximum tolerated dose that can be used in the second phase to assess damage to the genetic material.

During these studies the animals will be observed regularly to monitor changes in appearance and behaviour and an assessment of their body weights and amount of food they consume will usually be made to assess any potential effects that the test substance may have on the animals' general wellbeing. Blood samples will typically be collected to assess concentrations of the test substance in the body (in either the animals used to assess toxicity or in separate satellite animals (dedicated to providing blood samples), depending on the volume of samples required), and to assess any changes in blood components. Tissue samples will typically be examined to evaluate any changes in their weight, structure and function (and may occasionally be used to assess concentrations of test substances in them). For genetic toxicity investigations blood or tissues will be analysed for evidence of genetic damage.

Occasionally, based on knowledge of the test substance being studied, other measurements may be required such as analysis of urine, measurement of body temperature and an examination of the animal's eyes. Occasionally, information will be generated to develop or improve methods in our studies leading to 3Rs benefits. The measurements collected during preliminary studies are similar to those widely accepted by regulatory authorities to assess the toxicity of potential new medicines in regulatory studies. Prior to conducting preliminary general toxicity studies information will typically have been generated to ensure that a substance has the desired qualities to be considered suitable for development. This may include information from the use of predictive computer software packages, the literature and early assays in cells to predict safety concerns that may be associated with the substance or its mechanism of action. A limited assessment of how the substance persists in the body over time is also typically available and in some cases data from other studies conducted using the same substance or with similar substances may be available to support dose selection.

A tiered approach will be used for evaluation of genetic toxicity. A literature-based assessment is initially performed to identify potential issues that might occur, and expert computer systems will be used to determine if there are any concerns that the chemical structure of the test substance could cause toxicity. This information can also be used by drug discovery teams to improve the quality of future test substances. There is a strong correlation between a test substances ability to cause genetic toxicity in bacterial cells and cancer in rodents so if a test substance does cause genetic toxicity in bacterial cells it would typically be stopped from development prior to the use of animals. Test substances that show potential genetic toxicity in tests using mammalian cells or when there is concern about how they work in the body, may be assessed in preliminary genetic toxicity studies in rodents to understand if genetic toxicity might be produced in humans.

Regulatory Authorities generally require characterisation of toxicity in regulatory studies at maximum tolerated doses or doses that are large multiples of the levels of test substances anticipated to be used in humans. Since studies conducted under this project may be needed to support subsequent regulatory studies it is therefore generally necessary to perform them at high doses that may produce clear signs of toxicity. This may cause the animals to show clinical signs, reduced body weight gain or body weight loss and/or reduction or cessation of food consumption.

The scientific endpoints are those considered to be the minimum required to achieve the study objectives. 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 the toxicity of test substances 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?

There is a mandatory regulatory requirement to characterise the toxicity of potential new medicines in mammalian species (rodents and non rodents) during the programme of regulatory studies. Preliminary toxicity rodent studies need to use the same rodent species

intended for the regulatory programme and accepted by Regulatory Authorities, 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 risks of giving a potential new medicine to humans experiments will normally be conducted in conscious young adult animals with complex, integrated and well-developed physiological mechanisms and organ systems representing those of patients.

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.

All animals will be monitored closely after dosing with the frequency of observations related to the nature and intensity of any adverse effects observed. 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 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 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.
- Regular attendance at our scientific and ethical review forum by representatives from research statistics as part of the scientific and ethical review of studies.
- Introduction of enhanced caging for a study with an extended duration. This will be considered for future studies where applicable.

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:

- Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).
- NC3Rs - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies (2019).
- LASA - Guiding principles on good practice for animal welfare and ethical review bodies. (2015)
- Prescott MJ, Lidster K. Improving the quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46:152-156, (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).
- Smith A et al (2018). PREPARE: guidelines for planning animal research and testing. Lab Anim; 52(2):135-141.
- 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.
- Guideline limit volumes for dosing animals in the preclinical stage of safety evaluation (ABPI) RM Hull, 1995, Human and Experimental Toxicology, 14, 305-307.
- ICH Harmonised Tripartite Guideline M3 (R2) ‘Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals' (2009).
- ICH Harmonised Guideline M7 (R1) ‘Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk (2017)
- ICH Harmonised Tripartite Guideline S2 (R1) ‘Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended For Human Use’ (2011)
- ICH Harmonised Tripartite Guideline (S3A) 'Note for guidance on Toxicokinetics: The assessment of systemic exposure in Toxicity studies' (1994).
- ICH Harmonised Tripartite Guideline S6 (R1) ‘Preclinical Safety Evaluation of Biotechnology- Derived Pharmaceuticals’ (2011).
- ICH Harmonised Tripartite Guideline (S9) 'Non-clinical Evaluation for Anticancer Pharmaceuticals' (2009).
- Guideline on repeated dose toxicity. Committee for Human Medicinal Products (CHMP), European Medicines Agency (EMA). 2010. CPMP/SWP/1042/99 Rev 1 Corr.
- Guideline on non-clinical local tolerance testing of medicinal products. Committee for Medicinal Products for Human Use (CHMP), European Medicines Agency (EMA). 2015.
- EMA/CHMP/SWP/2145/2000 Rev. 1, Corr. 1
- Guidance on dose selection for regulatory general toxicology studies for pharmaceuticals. NC3Rs 2009
- The in vivo Pig-a assay: A report of the International Workshop On Genotoxicity Testing (IWGT) Workgroup. 2015. Mutation Research, 783 (1), 23-35.

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.

67. Connectivity and plasticity of developing and mature central nervous system circuits

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neurobiology, Synapse formation, Synaptic plasticity, Development

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 neuronal circuits in the brain form and are modified by experience.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work carried out here will not only enhance our understanding of how the brain is built during development but also provide insights into how organisms can adapt to their environment. This work may also shed light on key events that are compromised in neurodevelopmental disorders, such as autism and epilepsy, and may identify targets for future treatment.

What outputs do you think you will see at the end of this project?

The main outputs form this work will be in novel findings regarding the how brain forms, matures and adapts to its environment. The general understanding of the developmental processes that lead to the correct formation of our functional circuitry in the brain is essential to comprehend the complex brain functions such as memory, learning and behavior and makes it one of the fundamental issues in developmental neuroscience. But it is also important for processes involving axon regeneration after injury or disease and for understanding the mechanisms of neurodegenerative diseases. Notably, studying the mechanisms of neural circuit assembly will also help to gain insights into the basis of a range of mental disorders, such as Schizophrenia, bipolar disorder or autism, where links to neurodevelopmental causes, such as the establishment of axonal pathways or synapse formation have been established. In addition, this research has important links to epilepsy research, where circuits become hyperactive and loose stability.

Who or what will benefit from these outputs, and how?

The general understanding of the developmental processes that lead to the correct formation of our functional circuitry in the brain is essential to comprehend the complex brain functions such as memory, learning and behavior and makes it one of the fundamental issues in developmental neuroscience. We expect this work to lead to a greater understanding of the normal developmental processes that occur during synaptic and circuit formation in the developing brain, and how these processes are modified by the environment or perturbed in models of neurodevelopmental disorders such as autism spectrum disorder, epilepsy and schizophrenia. It will also establish *in vitro* and *in vivo* systems that we will use to test novel pharmacological agents.

In the short-term our findings will be useful to better understand the basic mechanisms behind the brain development and plasticity. This will mainly benefit scientists in the field. In the longer-term, we anticipate that our findings will also have an impact on clinical applications. The close links of this research to neurodevelopmental disorders could lead to the identification of potential cellular/molecular processes that can act as targets for drug treatments. We therefore anticipate that our findings may have an impact on clinical applications.

How will you look to maximise the outputs of this work?

We will make our data available first as preprints in BioRxiv and then through peer-reviewed scientific journals that can be accessed through PubMed, allowing our main findings to be monitored for accuracy and content. In line with current policy we will also ensure that data are published in journals with an open-access policy of not more than six months. Any reagents produced will be made freely available to the scientific community immediately on publication. We plan to publish and/or present our data at meetings.

Species and numbers of animals expected to be used

- Rats: 490
- Mice: 11600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 do these experiments in rats and mice. It is critical to achieve a balance between relevance to humans and sentience, and we feel that rodents offer the best compromise. The regions of the brain that are known to be important for neurodevelopmental disorders are reflected in rats and mice, and choice of model can be validated with relevant behavioural phenotyping (which, although caveats apply, are more relevant than, for example, in invertebrate species, and impossible in purely in vitro approaches). Further, mice are ideal due to the number of transgenic mice available including disease- relevant mutations and reporter lines, and increasingly transgenic rats will be available as well. Although currently there is limited availability of transgenic rats, rats have a wider repertoire of behavioural tests available and show better pharmacological alignment with humans so will increasingly will be the model of choice. The availability of inducible and conditional mouse lines allows specific targeting of developmental stages and cell types/circuits, which also minimises adverse effects. Additionally, in collaboration with molecular biologists, we can reduce or overexpress candidate molecules and examine their effect on the development of connectivity. Working with rats and mice also builds on the wealth of knowledge and research already available and minimises unnecessary repetition.

Typically, what will be done to an animal used in your project?

Animals in this project will mostly be transgenic animals (Protocol 1) where the expression of specific genes, some of which can be induced (Protocols 2 and 4), will allow us to either visualize or modify the activity of neurons but will have no harmful side-effects. In some instances, animals may undergo surgeries to either introduce genes or cells into the brains of mice at the embryonic (Protocol 3) or post- natal (Protocol 5) stage. In addition, to establish the role of neuronal activity on the development of the brain, we will also alter neuronal activity in animals during development to assess the role this plays in the formation of neuronal circuits in the brain (Protocols 1 and 5 to 7). Using tools that allow the visualisation and modification of neuronal circuits we will then measure the properties of neurons and the networks that they form (Protocols 6 and 7). This will initially be done in either in vitro or ex vivo systems for which mice will be sacrificed and the neurons from the brain removed to assess their properties (Protocol 6). Alternatively, neurons and their circuits will be imaged in vivo (Protocol 7).

What are the expected impacts and/or adverse effects for the animals during your project?

There is a low risk of complications that may arise from the anaesthetics and surgeries performed in this project and an equally low risk of post-surgical infections. Both will be monitored and minimised. The delivery of genetic and cellular material into embryos and post-natal animals are not expected to exhibit any harmful adverse effects, other than complications that may arise from the anaesthetics and surgeries described above. Finally, the in vivo imaging of neurons in the brain will require the implantaiton of a small cranial window that is not expected to exhibit any harmful adverse effects other than those that may arise from the anaesthetics and surgeries.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The procedures described here will be mild (Protocols 1) or non-recovery (Protocol 6) and will never exceed a moderate severity (Protocols 2, 3, 4, 5 and 7).

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 the fundamental processes underlying the development of synaptic and circuit development of the brain, and how they are modified by experience, we need to be able to manipulate the expression of genes and environment, as well as conduct invasive experiments that are not possible to do in humans. Mice provide an excellent mammalian system for the type of experiments proposed here. Mice share 95% of their genes with humans and genes linked to diseases in humans have also been shown to cause similar deficits in mice. They are relatively easy to maintain, reproduce rapidly and there are many transgenic animals that help scientific research. As a result, mice have provided an advantageous model system due to their close genetic and physiological similarities to humans, as well as the ease with which their genome can be manipulated and analysed.

Which non-animal alternatives did you consider for use in this project?

We have considered a number of non-animal alternatives. In fact, we will conduct a significant amount of the proposed work in vitro and ex vivo, predominantly in acute or cultured brain slices and cultured dissociated neurons, which will complement our study. In addition, we will also include using human iPSC-derived neurons and grown in vitro. Finally, we also considered the possibility of using zebrafish, a model system that we have used in previous collaborations.

Why were they not suitable?

Although in vitro and ex vivo rodent neurons will be useful in some mechanistic studies, to fully understand mechanisms that underlie normal development and how these are changed by experience, we will need to conduct work in vivo as well. We are also experimenting with the use of human iPSC- derived neurons grown in vitro but we find that this technology is not reliable yet and shows a large variability when assessing the formation of neuronal networks. Although promising, it is still an emerging technology with many caveats that need solving. Finally, although we have also considered using zebrafish as a possible system, we find that in many cases they have very different neuronal morphologies to rodent neurons (eg: most axons in the visual tectum originate from distal dendrites) that make it harder to interpret our findings.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 calculated based on the known or anticipated success of surgical procedures, biological variability and measurement variability. For example, experiments relying on repeated imaging of neuronal structures during development require a number of experimental steps with relatively low chances of success. Implantation of chronic imaging chamber is successful on 80% occasions (due to bleeding, damage to dura, or animals failing to recover fully from surgery). Before the animals enter a chronic imaging protocol, they are allowed to recover from surgery. During this time, bone growth, thickening of dura, and/or the incidence of infection can obscure the transparency of the imaging window which is essential for obtaining high-resolution images with a two-photon microscope. Only about 35% of successfully implanted animals pass this stage. As chronic imaging continues, the
windows of more and more animals grow over due to bone growth), such that on average only about 15% of the successfully implanted animals outlast the imaging protocol. Moreover, because imaging is carried out in animals expressing fluorescent proteins in a subset on neurons in the brain, the likelihood of finding labelled cells in the correct brain regions is about 50%. Thus the total success rate is 80% x 15% x 50% = 6%. It follows that for a control experimental group, data from at least 10 successfully imaged animals is necessary to carry out statistical comparisons (i.e. in this example, 10 / 6% = 167 animals requiring surgical implantation). In addition, the delivery of genes/cells/labelling agents to the brain has a limited success rate (about 75%), which further reduces of carrying out a experiment to its full completion. All these parameters were taken into account when assessing overall animals numbers needed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The proposed experiments will generally use factorial experimental design, which will maximize the data collected from each animal. We will use the appropriate statistical methods for analyzing the data (largely MannWhitney or ANOVA), and seek statistical advice as necessary. All experimental groups will be randomised and blinded where possible, and analysis of data will be done blind and/or automated. As soon as preliminary data is available to calculate effect size (Cohen’s d) appropriate power calculations will be undertaken to optimise sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

By doing much of our work in vitro or ex vivo experiments in tissue, either following schedule 1 or terminal anaesthesia, we will be able to obtain data from multiple cells and/or tissue slices to maximise data obtained from each animal. Further, we will use

tissue from transgenic animals of both sexes and all genotypes post-schedule 1, meaning we will generate far more information without any additional numbers of animals or suffering. Where specific brain regions are used we will store any remaining tissue for future use, to prevent unnecessary animal use. Where possible we will use internal controls (eg unaffected brain hemisphere) or paired 'before and after' design (eg for drug studies) to maximise power and minimise numbers.

We will minimise variability by using inbred strains of mice, housed in standard conditions, ensuring careful matching of age and standardisation of experimental protocols, including good breeding colony management. The use of live imaging will also allow a reduction in the number of animals used as data from sequential times is obtained, rather than using different 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.

Choice of species/model:

We will do these experiments in rats and mice. It is critical to achieve a balance between relevance to humans and sentience, and we feel that rodents offer the best compromise. The regions of the brain that are known to be important for neurodevelopmental disorders are reflected in rats and mice, and choice of model can be validated with relevant behavioural phenotyping (which, although caveats apply, are more relevant than, for example, in invertebrate species, and impossible in purely in vitro approaches). Further, mice are ideal due to the number of transgenic mice available including disease- relevant mutations and reporter lines, and increasingly transgenic rats will be available as well. Although currently there is limited availability of transgenic rats, rats have a wider repertoire of behavioural tests available and show better pharmacological alignment with humans so will increasingly will be the model of choice. The availability of inducible and conditional mouse lines allows specific targeting of developmental stages and cell types/circuits, which also minimises adverse effects. Additionally, in collaboration with molecular biologists, we can reduce or overexpress candidate molecules and examine their effect on the development of connectivity. Working with rats and mice also builds on the wealth of knowledge and research already available and minimises unnecessary repetition.

Choice of method/minimize suffering:

The methods outlined in the protocols in this application have been carefully chosen to allow us to achieve our objectives while minimising animal suffering. The vast majority of animals will only undergo a single procedure, and much of the work will be done in fixed tissue or in vitro / ex vivo using tissue obtained after Schedule 1 or terminal anaesthesia. All animals undergoing surgery will be given analgesics to minimise suffering and the

electroporation system we will use has been shown to result in optimum survival and minimal tissue damage. Further, many of our preliminary experiments will be conducted either in cell culture or in live tissue taken from wild type rodents which will enable us to plan experiments and minimise animal usage and suffering. Finally we aim to implement a variant on the standard methods of in vivo imaging that is much less invasive and therefore represents a refinement of technique.

We do not plan to use any protocols categorised as severe.

Why can't you use animals that are less sentient?

This work centres around the development and plasticity of neurons in the brain. In particular, these studies focus on understanding how sensory inputs can modulate brain development and are also linked to developmental disorders, such autism, schizophrenia and epilepsy. As a result, we need to work on animals that are closely related to humans but where we can also perform invasive experiments. Mice provide an excellent mammalian system for the type of experiments proposed here. Mice share 95% of their genes with humans and genes linked to diseases in humans have also been shown to cause similar deficits in mice. They are relatively easy to maintain, reproduce rapidly and there are many transgenic animals that help scientific research. As a result, mice have provided an advantageous model system due to their close genetic and physiological similarities to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will make sure that animals undergoing a procedure undergo the best care possible. This includes a constant re-evaluation of post-operative monitoring and care to minimise welfare costs following a procedure. In parallel to this, we include in each protocol, stringent pain management procedures to further decrease discomfort that we have developed over the past 5 years. These procedures will be regularly re-evaluated an updated to reduce harm as much as possible.

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 the current literature to ensure our experiments are designed optimally. Individual experiments will generally involve factorial designs, to maximise the information gathered using the minimum number of animals. For the design of complex experiments, advice of a statistician will be sought.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will follow, as we regularly do, the 3Rs section of the NC3R website. This site provides all the latest information and advances in this area.

68. Breeding and Maintenance of GA poultry lines

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Breeding, Maintenance, Poultry

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?

The overall aim of the project is to breed and maintain genetically altered poultry lines. This licence will act as a service licence to facilitate the provision of genetically altered eggs, embryos and birds to other research projects. By providing these valuable tools, a wide range of scientific research will be facilitated.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chickens, eggs and embryos can provide a valuable model for a wide variety of scientific research programs. Globally the chicken is the most numerous agricultural animal. Gaining greater knowledge of the species has important implications for poultry and human health and can contribute to global food security. Better understanding of the species can be utilised to improve welfare standards of farmed chickens. By breeding birds on this licence, a wide range of research will be possible.

What outputs do you think you will see at the end of this project?
This purpose of this project is to support the research goals of institute and greater scientific community. The measurable outcome of work carried out will be the provision of

GA eggs, embryos and chickens.

New information and publications subsequently arising from use of this material are likely be high given the diversity of projects which will be supported. Lines held under this licence have provided the basis for greater understanding and publications in developmental biology such as eye and limb formation.

Development and structure of cells and organs have also be strongly aided by these lines.

Who or what will benefit from these outputs, and how?

Material generated under this licence will be supporting a wide range of projects including chicken physiology, immune function, vaccine development and productivity. The potential benefits will be relevant to be human and chickens. Poultry disease can have severely detrimental effects upon commercial and subsistence farmers. Through greater understanding of the chicken and its' immune system, there is potential to reduce disease incidence and increase food security.

Agricultural companies will potentially benefit as their production costs though greater understanding of the birds and how to increase their production capabilities.

How will you look to maximise the outputs of this work?

By its nature, this project is supportive to and reliant upon a collaborative approach. Detailed records of material supplied internally and externally to the institute will be kept. Details of successful publications for institute staff and students are collated and displayed on an external facing website. Other initiatives such as the recently held Avian Research Symposium (Feb. 2021) encourage information sharing and collaboration. Strong links with external researchers utilising material generated from this project will help to share positive and unsuccessful lines of enquiry.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 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 main driver for production of GA chickens specifically is the downstream requirement for material for use in related projects. Utilising the chicken is a valuable model. Study of GA eggs and embryos can allow research, such as developmental biology, to take place at a very early developmental stage. The benefit, when compared with mammalian models, is the ease and non-invasive way in which the egg/embryos are collected. Juvenile and adult birds are required for projects such those studying the immune system and vaccination effects.

Typically, what will be done to an animal used in your project?

The main remit of this licence is the provision of GA embryos, eggs and chickens. Given the scope, the licence will be minimally invasive. Blood samples may be taken to confirm the presence and the nature of the genetic alteration. Birds will generally be group housed with natural mating allowed to take place. Natural mating may be supplemented with artificial insemination should it be required.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the birds bred under this licence will not display any detrimental effects as a result of the genetic alterations. A smaller proportion of birds may display behavioural and physiological changes due to carrying the alteration. For the most part, any changes are limited to lethargy, reduced weight gain.

Within strains affected by the carrying a genetic alteration, raised mortality against the background strain has been noted. Birds or bird lines which are negatively affected by GA will have a clearly defined care plan.

Birds, though not all, will be blood sampled which may result in localised bruising. Bruising which occurs generally clears over the course of a week.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority will be subject to a mild severity limit with many individuals falling into the subthreshold category with a smaller number falling into the moderate category. Proportionally, the ratio of birds falling into the two categories is expected 80% and <20% respectively for mild and moderate.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?
The chicken is a globally significant food source with commercial farmed chickens outnumbering humans. Many complex body systems cannot be studied without directly looking at the birds themselves. For birds which are supplied to vaccination and infection studies, observations and data gathered directly from chickens is necessary. When considering developmental biology, visualising developing cell populations and organ development can give unique insights. Use of early age embryos generated under this project will allow other projects replace protected animals are therefore reduce overall numbers.

Which non-animal alternatives did you consider for use in this project?

Given the remit of the licence, there is difficulty in considering suitable alternatives to using an animal model. Dialog with end users will be ongoing and any alternative models which become available for their specific project communicated. Though, it is expected that anyone using chickens bred on this licence will first robustly then continuously seek non-animal models.

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?

Previous utilisation of eggs, embryos and birds has been used to inform the estimated numbers within this project. Within each breeding line, numbers will be calculated by taking into consideration the output required and the minimum number of individuals required to maintain a healthy population.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Care and consideration will be used to create bespoke breeding plans for all individual lines. Generally, GA birds bred on this licence have been created on a commercial hen background. Commercial hens will be raised in the unit and periodically interbred with GA lines. By crossing our GA lines with a commercial line, the number of individuals required to maintain a healthy population is greatly reduced.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

By ensuring efficiency in all steps of incubation, hatching and rearing, the number of individuals required for egg production is reduced. Where birds are generated to provide tissue samples, sharing of resources will be strongly encouraged to reduce the overall number of birds 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.

Birds will be naturally mated and eggs collected daily. In some cases artificial insemination may be required. In the majority of the chickens, there will be no apparent effect from the genetic alteration. Genotype may need to be confirmed prior to assigning new breeding groups. By using hatching shell waste to determine genotype, this can reduce the need blood sample live birds. All chickens will be held to home office guidelines in floor pens with bedding material to encourage display of natural behaviour.

Why can't you use animals that are less sentient?

Chickens supplied for use within additional projects will only be supplied if the lack of suitable alternative has been robustly demonstrated.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Prior to arranging new breeding programs, where possible, genotype will be determined using egg shell waste negating or reducing the need to blood sample. As procedures other than blood sampling will be undertaken on this licence, the main refinement will be though the promotion of good husbandry practices. Most birds bred will not display any negative effects due to the genetic alteration. Lines which do display an effect will have bespoke care and welfare plans which will be well documented.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All animals will be kept in accordance with the Home Office Code of Practice, though the majority of chickens will be afforded greater space than the minimal requirements. Close communication with the NVS and the Poultry Welfare Taskforce will allow communication of technique development.

Procedures carried out under this project will be undertaken by the dedicated poultry teams, competencies in techniques will be regularly considered to ensure best practice. The NC3Rs website will be accessed with regard to efficient breeding strategies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Care of animals created within this project will be undertaken by our dedicated poultry husbandry and technical staff. There is very strong communication between the poultry team and both Named Veterinary Surgeon and the Animal Welfare Ethical Review Body. The NC3R website will be regularly visited to ensure effective communication of new information. To further promote welfare and best practice, a recently set up Poultry Welfare Taskforce will spearhead raising standards and promoting the 3Rs within our poultry facilities. Methods for creating new lines with reduced incidence of negative effects are being developed within the institute, close collaboration with researchers will allow communication of new developments.

69. Xenopus as a model for vertebrate development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Xenopus, develomental biology, Stem cell biology, cancer

Animal types	Life stages
Xenopus laevis	adult, embryo
Xenopus tropicalis	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 this project is to understand how animals develop. It is difficult to study development in humans as the embryo develops inside the mother and gestation can take up to 9 months. Scientists therefore use animal model systems such as Xenopus (the African claw toed frog), which are easier to study to look at developmental events.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Knowledge gained from research in model organisms can be applied to human development especially as over the last 100 years it has been clearly shown that many developmental processes are conserved between humans and the rest of the animal kingdom.

In order to assess a genes function, a rapid way of testing whether mutation of the gene

causes a specific phenotype which could be linked to specific disease is required. While primary cell cultures, organoids and biochemical studies are informative in some cases, these cannot reflect the complex interactions that occur within tissues, organs and whole organisms. Although mammalian models can be used, the ethical and financial cost is high so alternatives are sought. Of these, Xenopus is most highly related to mammals and alone shares its genome architecture with humans. Data from labs around the world has shown that Xenopus is an effective model in which to make and test mutations of specific genes in orer to better understand their function in higher orgnaisms.

What outputs do you think you will see at the end of this project?

At the end of this project we would hope to have a better understanding of many aspects of developmental biology. This will be evidenced by the publications of papers in relevant journals and the generation offunding for proposed projects.

Who or what will benefit from these outputs, and how?

In the short term the beneficiaries of our work will be students and other researchers trained in the lab to be the scientists of the future.

The identification and characterisation of developmentally important genes will be of benefit to developmental and cell biologists studying basic embryology. They will be able to integrate the data about the function of these genes into the genetic regulatory networks that drive development. This will lead to a better understanding of developmental processes.

In the medium to longterm our research will be of relevance to understanding the causes of and treating human conditions such as developmental abnormalities, genetic abnormalities, cancer development and metastasis. Our work will have the potential to help others to develop novel treatments or preventatives for such conditions?

How will you look to maximise the outputs of this work?

I will endeavour to publish the groups work wherever possible in appropriate journals. Myself and others in my lab will attend relevant conferences and present our work in the form of invited talks or posters.

Species and numbers of animals expected to be used

- Xenopus laevis: 7500 tadpoles - before protected age (st.45), 125-200 adults
- Xenopus tropicalis: 1800 tadpoles - before protected age (st.45), 50-100 adults

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We are using the tadpoles of Xenopus laevis (the African clawed frog) and Xenopus tropicalis as model organisms. We use Xenopus because the females can be induced by a simple injection to lay eggs all year around when kept in an ideal environment and this

provides us with a source of embryos (frog spawn). During the course of a year a colony of approximately 150-200 frogs will be induced to lay eggs 1-3 times. The better the quality of care of the frogs the better the quality of the eggs/embryos they produce. To therefore minimize having to repeat experiments it is essential to have healthy animals.

The Xenopus embryos develop outside the mother and they are large which makes observation and manipulation relatively easy. Tadpoles have hearts, livers, brains, eyes and kidneys just like humans. In fact a tadpole with a fully functioning heart, which can be observed beating using non-intrusive methods, develops from a fertilised egg within 4 days. Embryos produced will be used to study questions of cell communication and migration using a variety of methods. Therefore, what we learn in tadpoles about basic cellular and molecular developmental mechanisms will enhance our understanding of development in humans especially with respect to abnormal or disease situations.

Typically, what will be done to an animal used in your project?

Female frogs from whom we need to obtain eggs are injected with PMSG 4-7 days before the eggs are needed in order to prime the animal to generate good and healthy eggs. The day before an experiment the females are further injected with HCG (Human Gonadotrophic Hormone) which induces them to lay their eggs 12-18 hours later. Each female is very gently massaged to get her to lay her eggs in a petri dish. This procedure is carried our 1-6 times over the course of a day with a minimum of 1 hour rest between squeezes. The female is then observed for a further 12-18 hours to make sure they are fine before being returned to the frog facility.

Males (laevis and more commonly tropicalis) are injected with HCG to make them more fertile before they are sacrificed and the testes removed in order to carry out in vitro fertilisation of the eggs.

Note - Commercialy bought Frozen sperm are increasingly being used which means that over time the number of Males being used will decrease.

With respect to embryos generated we will be manipulating genes by loss of function methods (Morpholinos or CRISPR) or gain of function methods (injection of mRNA coding for a gene of interest) to determine their effect on the developing embryo.

What are the expected impacts and/or adverse effects for the animals during your project?
The procedures used are mild and next to no adverse effects are expected with the procedures used.

For any affected tadpoles there will be diverse adverse effects including potentially: heart or circulation defects, craniofacial defects, altered behaviour, gut defects limiting the ability to feed properly and there may even be alterations in overall body structure. Any tadpole showing adverse effects will be humanely killed. Only healthy embryos will be allowed to continue to develop.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of the procedures on the female Xenopus and male Xenopus during induction of laying of eggs and fertilisation of the eggs is mild. When used for natural mating the females and males can be reused after a suitable recovery time (minimum 3 months) They can be used in this way multiple times until they reach an age when they no longer produce eggs of a suitable quality (approx 10-15 years of age). At this time they are euthanized. The proportion showing an adverse effect is approx. 0.5-1%.

With respect to genetically altered tadpoles we envisage mild severity in 80-100% and they will be euthanized by stage 45 of development.

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 purpose of the proposed work is to obtain fundamental knowledge on the development of a vertebrate organism. This requires working with the whole organism.

Which non-animal alternatives did you consider for use in this project?

Tissue culture, organoid culture, computer simulations

Why were they not suitable?

In development responses and changes occur due to interactions between different cells and tissues. Cell based assays generally use only one cell type so are not a good model in this respect. Organoid cultures can sometimes include more than one cell type but are still limited and do not include a functioning vasculature.
Computer simulations are increasingly being used in development but in all cases in vivo experiments do at some point need to be done to validate and test predictions and 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?

These are based on the numbers needed from past experience and from preliminary experiments, which have effectively been pilot studies for this investigation.

Tadpoles: For each gene we are studying we will keep 50 tadpoles up to the protected age in each experiment, this allows for the natural death rate of tadpoles and to have 30 animals for phenotype analysis. We perform 3 biological replicates of each experiment. For the actual test experiments we will re-create 50 mutations, each requiring 150 tadpoles; 7500 tadpoles in total. In some cases where we are generating founder lines for specific mutants or reporters healthy individuals (approximately 50 tadpoles in total) will be kept until adulthood.

Adult Animals: When the lab is running at maximum efficiently we plan to do 1-2 Frog experiments a week. One frog experiment involves priming, inducing and collecting the embryos from 3-6 frogs. We usually collect eggs most weeks (approx 40 weeks a year). Frogs can be used after a minimum of 3 months. Therefore over a year we would use up to 12 frogs per week for 40 weeks = 480. Frogs can generally be reused 3 times in a year so 480 divided by 3 = 160. So at maximum efficiently we would look to use 160 female frogs in a year.

We do fewer Xenopus tropicalis experiments (approx 20 per year) so need fewer frogs and use fewer tadpoles.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each week we have a lab meeting of all the Xenopus users at which we determine what people are planning for the following 1-2 weeks. This way we can plan experiments where personnel share the eggs/embryos produced and so maximise the efficiency of one experiment. Occasionally experiments will be delayed or bought forward in order that one day of egg collection can be carried out instead of two.

At this current time we are transitioning to using commercially bought frozen sperm instead of live males for in vitro fertilisation of eggs. This will not fully replace using Males but will drastically reduce the number of Males we do use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our Xenopus facility is designed to keep the frogs as healthy as possible. The healthier the frog the more likely she will produce good quality eggs. If we can be sure of obtaining good eggs we can reduce the number of females we inject for each experiment. We are continually looking for ways to improve the care of the frogs in a cost effective manner. We are planning over time to initiate the chipping of the frog colony. This will enable detailed records to be kept for each frog. those that routinely provide bad quality eggs will be culled to improve the efficiency of the system.

The Xenopus community in the UK and the rest of the world routinely discusses issues of care and welfare for the animals at meetings such as the British Xenopus group meeting and once a year for the International Xenopus meeting or Xenopus PI meeting in the USA. Web sites such as http://www.xlaevis.com/ also provide updated information. I am in routine contact with colleagues elsewhere in the UK and the rest of the world who provide help and assistance on best practice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Xenopus laevis, Xenopus tropicalis We will use Xenopus because:
Large numbers of Xenopus embryos are produced externally and obtaining them requires only a mild procedure (injection of HCG into the female).

We will use tadpoles prior to metamorphosis, when the brain is poorly developed and lacks the region that, at least in man, generates the distress associated with pain. The tadpoles are largely transparent, allowing simple observation of many of the phenotypes produced without invasive methods.

Why can’t you use animals that are less sentient?

We are studying vertebrate development and require embryos to do this. Xenopus laevis and Xenopus tropicalis embryos/tadpoles develop outside the mother which is beneficial compared to mice.

The frog's genome is very similar in structure and detail to the human one and more than 80% of the currently-identified human genetic disease genes are found in it. To use a less sentient animal in which the genome is sufficiently well annotated for these studies means using zebrafish or Drosophila. The latter, in many cases, lacks sufficient protein identity to humans to make it possible to re-create human disease phenotypes. The structure of the zebrafish genome is very different from that of humans/frogs.

In terms of developmental stage, we use the earliest possible for all experiments but have to judge this on a case-by-case basis depending on the cell type or organ affected by introducing the mutation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We mainly propose to use Xenopus laevis and Xenopus tropicalis for our experiments as they are a well characterised and well used model organism for vertebrate development. They are relatively easy to keep and the adults are subjected to only mild procedures.

As mentioned before we are gradually transitioning to using frozen laevis and tropicalis sperm for in vitro fertilisation. This will in time lead to needing less males in our facility.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Online protocols are published by many Xenopus labs and are often available on lab websites. Xenbase and the Xenopus Stock Centres in the UK and the United States also publish standard protocols. Online forums and the biannual International Xenopus Meeting and Xenopus PI Meeting also allow for free exchange of knowledge and best practice.

In addition Cold Spring Harbor Protocols publishes may papers in the 'Xenopus Collection' (http://cshprotocols.cshlp.org/cgi/collection/xenopus) which provide an up to date resource.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am in regular contact with colleagues in the UK and the rest of the world from whom the latest news and advances in Xenopus research can be learnt. The Xenopus community has an online news magazine called Xine from which news is obtained. In addition the Xenopus community in the UK and the rest of the world routinely discusses issues of care and welfare for the animals at meetings such as the British Xenopus group and once a year for the International Xenopus meeting or Xenopus PI meeting in the USA. Web sites such as Xenbase (http://www.xenbase.org/entry/) and http://www.xlaevis.com/ also provide updated information.

70. The role of hormones, genes and diet in diabetes 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

Key words

Diabetes, Genes, Diet, Metabolism, Therapy

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 the project is to understand how interactions between hormones, genes, environment and diet lead to diabetes in rodent models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Type 2 diabetes mellitus (T2DM) currently affects ~5% of the UK population, with a rising incidence. Patients with T2DM do not live a normal healthy lifespan, instead suffering a number of complications and living on average 10 years less than someone without the disease. Complications include blindness, amputation, nerve problems, kidney problems, as well as increased risk of developing cancer and heart disease. Despite this, the

mechanisms underlying the onset of T2DM are still not well known. This might be in part because T2DM research receives just a fraction of the funding devoted to cancer and heart research. At its most simplistic, T2DM occurs in genetically susceptible individuals exposed to a permissive environment (e.g. diet, lack of exercise). Over the last two decades, rodent models have made important contributions to our understanding of how genes and environment interact to cause T2DM. This is because we can precisely control the environment of rodents, as well as manipulate specific genes. Thus, rodents have uncovered how risk genes contribute to T2DM, and have shown how diet and obesity influences the development of the disease. Moreover, 'preclinical' rodent models have been used to test responses to diabetes therapy, with a number of these treatments now used widely in the clinics (e.g. incretin-mimetics). The present project aims to use the latest rodent models of diabetes to provide new insight into how genes and environment influence T2DM, and how this can be treated. The results are directly relevant for human medicine and will go on to inform new treatments for T2DM.

What outputs do you think you will see at the end of this project?

The studies will identify a number of new genes that contribute to T2DM risk. In addition, the effects of environment, in particular diet, upon gene function will be better understood. This new information is important for understanding why some people develop T2DM, why others don't, and how we treat the disease. New findings will be published in the scientific literature, where they can be accessed by others. We will also work closely with stakeholders (i.e. patients with T2DM and the general public) to present our findings, for example through online webinars, lab tours and involvement in research planning. Should we identify genes with direct relevance for drug therapy, we will protect the intellectual property before working to translate the findings into human benefit. Lastly, we anticipate the development of new mouse strains which will: 1) provide new information about T2DM and how can we treat it; 2) be used in drug development programmes; and 3) reduce the number of animals used in drug testing (e.g. by allowing us to screen beta cell mass non-invasively and longitudinally).

Who or what will benefit from these outputs, and how?

Publication of new results and animal models will be important for others who may want to adopt similar approaches (3 years). Development of animal models for more refined drug screening is attractive, since methods to longitudinally measure responses to treatment are more powerful and require fewer animals (5 years). The general public will benefit over the longer term, since the project will provide a better understanding of the causes of T2DM (10 years). Finally, clinical trials or therapy may be informed by the studies here, leading to better outcomes for T2DM patients (10 years).

How will you look to maximise the outputs of this work?

Positive and negative results will be shared with the wider scientific and clinical communities through presentation at conferences, publication on free-to-view servers or open-access scientific journals. New techniques and protocols will be rapidly posted online and shared with the field. New animal models will be freely distributed to academics and industry so that they can be further validated, at the same time informing the results of our own work. Where the results have the potential to impact human health and wellbeing, we will seek to protect the invention so it can be brought to market. Lastly, we will also engage with the public, focusing on people with lived experience of T2DM.

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.

Mice will be used, since we need to perform genetic alterations for which this species is most amenable and well characterised. Moreover, the study is based on research already performed using mice. Mice will be studied as neonates and juveniles to understand pancreas development. Mice will be studied as adults, since this is the most relevant model for the study of T2DM and is reflective of the disease in humans.

Typically, what will be done to an animal used in your project?

Genetically-altered (GA) mice and their control littermates will be bred using established crosses and husbandry techniques. Most GA mice will possess the gene deletion/overexpression from birth, but in some cases we will need to administer a single injection or special diet to induce gene deletion/expression in adults (for example because the gene affects pancreas development). Some mice will develop diabetes on normal diet, but the majority will need to receive a diabetes-causing diet or diabetes-causing drug, typically delivered in the drinking water or via injection. Some mice will act as controls and receive normal diet or placebo. Diabetes progress is monitored by taking blood samples every 2-4 weeks to measure glucose/insulin/glucagon in response to glucose/insulin/pyruvate, as in humans. Mice will be treated for diabetes using drugs used in human medicine, administered in the drinking water, gavage or via injection. Responses to treatment are monitored by taking blood samples every 2-4 weeks to measure glucose/insulin/glucagon, as in humans. Mice may be humanely killed before and after diabetes induction and treatment, to allow detailed in vitro examination of the pancreas.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically-altered mice might spontaneously develop diabetes. Diabetes is well tolerated in rodents and is associated with excess urination. Blood sampling for glucose/insulin/glucagon is associated with mild discomfort and transient pain during the collection of samples. Administering diabetes-causing diets can lead to excess urination. Diabetes-causing diets can lead to skin irritation due to a greasy coat, although this is rare as animals are fed from bowls on the cage floor rather than from a hopper above. Administration of diabetes-causing drugs is associated with mild discomfort and transient pain due to injection. Administration of diabetes medication is associated with mild discomfort and transient pain due to injection. The estimated duration of these effects is 8-12 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)?

About 25% of mice will undergo a combination of procedures, like diabetes-causing diet, blood sampling for glucose/insulin/glucagon levels and diabetes treatment. This will be classed as moderate severity.

The remaining 75% of mice will only be used for breeding and/or will be used as tissue donors, and will not undergo a combination of procedures. This will be classified as mild severity.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We investigate how genes and environment contribute to the development of T2DM. This is a complex disease which perturbs many systems involved in regulating blood glucose levels and can only be examined in mammals. Indeed, humans and rodents regulate their blood glucose levels using conserved mechanisms and it is because of this that rodents have gone on to be the most widely used preclinical diabetes model.

Which non-animal alternatives did you consider for use in this project?

Primary and immortalized cultures, as well as computer simulations, have guided the studies proposed here and will continue to be used. Islet-like structures derived from human embryonic stem cells (hESC) have also been considered.

Why were they not suitable?

Cell lines cannot be used alone, since they lack proper cell-cell interactions present in the native pancreas and they are unable to replicate the in vivo mechanisms that regulate blood glucose levels. Computer simulations are helpful for setting parameters and modelling system dynamics, but rely on in vivo data as input and can’t replace in vivo models. Islet-like structures still function poorly, since the derived cells are not as mature as those in primary islets. Moreover, use of hESC presents its own set of ethical challenges.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

First, we define the exact measure that we will analyse (e.g. blood glucose, body weight etc). We then determine what the independent replicate is (each cell, each animal, each day?). Expected effect size (i.e. how big the change is) is determined through our previous studies, consultation of the literature, cell culture based in vitro analysis or through small pilot experiments when possible. Finally, from these pieces of information we can accurately calculate the number of animals required to produce a statistically robust result.

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 to design experiments and taken advice from researchers who have performed similar research projects in the past.

The minimum number of animals required to give a valid result has been calculated using careful statistical analysis. Moreover, animal use will be minimised by validating the majority of experiments firstly in vitro, or by using computer simulations to refine experimental parameters. Throughout, we take steps to reduce subjective bias, as well as undertake the most appropriate statistical analysis. In general, experiments will involve a factorial design that will maximise the information obtained from a minimal number of animals. Furthermore, data can be obtained from the same animal at different time points, which increases experimental power and reduces the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Inbred mouse strains will be used to minimise genetic variability and skilled technicians and lab members will be used for animal handling. Breeding strategies will be optimized to avoid animal wastage. Tissue not used in the present project (e.g. liver, gut) will be made available to other investigators who investigate T2DM. All tissue will be stored and made available to other researchers in the field, including posting of imaging and genetic datasets on public databases. A PREPARE guidelines checklist will be completed before each new experiment. Data from the experiments will be published in accordance with the ARRIVE guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Lower vertebrate models, such as zebrafish, are not relevant for the studies here, since they do not share the same mechanisms of blood glucose regulation to humans and rodents. Similarly, immortalized mouse and human pancreatic cell lines do not recapitulate the interactions that exist between different cell types in the pancreas. Stem cells from

humans can be made to form islets, but these do not function well, and therefore, one of the aims of our research is to understand why, using animal models. The scientific literature will be reviewed on a regular basis to identify any novel technologies and models that could be adopted to replace in vivo animal use. Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially adopted in order to replace in vivo animal use. As such mice present the most refined model to understand diabetes.

Glucose, insulin and pyruvate tolerance testing are similar to those used in human medicine and are less invasive than hyperinsulinemic-euglycemic clamps, which produce high quality data but require anaesthesia and implantation of catheters. While continuous glucose monitoring via a wireless implant is becoming available in humans, the technology is still in its infancy in small animals and is beset with a number of issues including inaccurate data capture and the need to surgically implant the monitor.
Nonetheless, we will keep abreast of innovation in this arena and switch once the balance of harm is less than the methods already in use. To minimise adverse effects during these tolerance tests, only sterile solutions will be injected, syringes will only be used once and a local anaesthetic (EMLA) will be applied to the blood sampling site. Hormone concentrations will be measured using ultrasensitive assays, minimising blood volume used. Where possible, animals are handled in cardboard tubes, which minimises stress. Animals are habituated to being handled for 1-2 weeks preceding any experiment. Physical restraint is only needed during the initial access to the vein to avoid harming to the animal. For animals on diabetes-causing diet, softer bedding and more absorbent material will be used to avoid excessive grooming. Food will also be provided in bowls on the floor to avoid distribution onto the animals and into the cage. Diabetes-causing drugs, labelling compounds and diabetes therapy are all based upon standardised protocols and the lowest doses will be used to induce a measurable effect.

Why can’t you use animals that are less sentient?

Lower vertebrates, such as fish, and invertebrates, such as flies, are not relevant here, since they regulate glucose differently to humans. Moreover, fish and flies are not amenable to metabolic phenotyping (hormone measures from blood are difficult/impossible), and do not possess islets of Langerhans like in mammals (rather a small organ of 20 or so cells). Foetal mice cannot be used, since their pancreas only develops fully after birth, specifically following the transition from milk to carbohydrate/protein-rich diet. Lastly, terminally anaesthetised mice cannot be used because anaesthesia influences hormone release and blood glucose levels (i.e. the primary measures here).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We keep abreast of and rapidly adopt any advances in the field which lead to refinement. Examples of this include:

Use of highly-sensitive hormone assays for insulin and glucagon that allow smaller blood volumes to be more reliably tested.

All animals may receive local anaesthetic cream before blood sampling, which decreases stress/discomfort and improves the reliability of hormone and glucose measures.

Where possible, mice are acclimatised to handling before conducting any measures. This

decreases stress, as evidenced by a reduction in basal blood glucose measures.

Where possible, mice are handled using cardboard tubes to avoid stress associated with being picked up by the tail.

Generation of animals that allow longitudinal monitoring of beta cell mass using minimally-invasive techniques.

Where possible, drugs are administered in drinking water, which leads to robust responses without the need for injections.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult the literature for experimental design, best practice, and humane endpoints for metabolic and diabetes research in animals. We will also consult Simon Bate's book, 'The design and statistical analysis of animal experiments', for experimental design, statistical analysis, and sample size calculations.

Prior to all experiments, we will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment (PREPARE: guidelines for planning animal research and testing.
Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017 Aug 3.PMID: 28771074).

The resulting data will be published in Open Access Journals wherever possible and in accordance with the ARRIVE 2.0 guidelines published by the NC3Rs.

The LASA guidelines: 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.)

Jones HRP, Oates J, Trussel l BA (1999) An applied approach to assessment of severity. In: Humane End points in Animal Experiments for Biomedical Research (Hendriksen CFM, Morton DB, eds).
London: Royal Society of Medicine Press, pp 40±7.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will perform literature search, attend vendor’s information sessions, seminars and conferences to find out about new technology and new approaches that we could implement.

We will comply with the ARRIVE guidelines 2.0 (Animal Research: Reporting In Vivo Experiments; www.nc3rs.org.uk/arrive), a NC3Rs-developed checklist of the essential information that should be included in publications reporting animal research. We will sign up the NC3Rs newsletter and attend relevant 3Rs events.

71. Breeding and Maintenance of Genetically Altered Animals for Regenerative Neuroimmunology Research

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Mouse Breeding, Genetically altered mice, Colony Maintenance

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 breeding, generation, and maintenance of mice with genetic modifications. These mice will supply project licenses within our group with the appropriate mice needed to carry out the experimental work.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When mice are used in the study of human diseases, they are frequently selected because of their similarity to humans in terms of genes, as well as brain structure and function. Additionally, mice are the most widely used animal species in experimental research. This is because it is relatively easy to induce disease models like human diseases, and to manipulate their genes (i.e., genetic modification) in a way that allows studying specific mechanisms of disease. Here, genetically-manipulated mice are

produced for the experimental licenses in our group. These will help us understand how cells of the immune system can affect the healthy, diseased, or injured brain's structure and function.

What outputs do you think you will see at the end of this project?

Outputs will be a steady supply of appropriate mice for studies on other projects.

Mice produced in this licence will be used to study aspects of the human disease multiple sclerosis and human spinal cord injury. These mice will help us understand how cells of the immune system and the brain talk to each other. The mouse behaviour will be studied.

New-born mice in this license will also be used to get immune system and brain cells to grow in lab dishes.

Who or what will benefit from these outputs, and how?

The projects receiving these mice will have a reliable source of mice to use for their experiments.

How will you look to maximise the outputs of this work?

Lab members will meet and design a breeding plan to make sure the right number of mice are produced to match the needs of the experiment. Mice with genetic modifications will be made available to other labs we work with. All mice used on projects carrying out experiments within our group will be described in our publications. If we make a mouse with a genetic modification that does not work as expected (i.e., what is generally defined a negative result), we will look to also publish the negative results as appropriate. The licenses this work is supplying will benefit from good maintenance of the colonies to supply the mice we need at the appropriate times. Lastly, we will look to share tissue (i.e., cells and tissue) with other groups as appropriate and as needed.

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 using mice because currently they are the most commonly used animal in human disease research.

We are using adult mice as they need to be sexually mature for breeding purposes. Adult mice will supply project licenses within our group with the appropriate mouse models needed to carry out the experimental work.

We are using new-born mice to collect cells to grow and keep in a dish to do experiments.

Typically, what will be done to an animal used in your project?

Female mice will be given substances to make more eggs that are collected in the mouse ovary (i.e., superovulation). Substances will be given across two injections approximately 48 hours apart.
Substances will be injected into the abdomen (i.e., intraperitoneal). Mice will be killed at the end.

Male and female mice will undergo surgical procedures using appropriate anaesthesia. For male mice, they will undergo a surgical procedure to render them sterile (i.e., vasectomy). For female mice, they will experience a single surgical procedure to place embryos, or fertilised eggs, into their reproductive tract. Female mice may also undergo non-surgical placement of embryos into their reproductive tract.

This involves inserting a small thin tube into the uterus of the mouse to insert embryos directly.

Male and female mice will be housed together and allowed to naturally mate. This will produce mice with and without genetic modifications using standard breeding methods.

Some mice offspring (<50 total offspring per year) up to five days old will be collected and will be killed by removal of their head (i.e., decapitation). We will then collect cells of from the brain. These cells will then be grown in the laboratory and used for experiments.

Adult female or male mice will first be terminally anaesthetized. Then we will remove the blood by flushing it out of the mouse using a pump. Then we will collect the organs and store them in a solution that prevents the tissue from being destroyed. This allows us to store the tissue for long periods of time. Then, we will use the tissue for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Female mice that are breeding, receive injections for superovulation, or undergo a non-surgical procedure are likely to experience mild pain (i.e., that equates to the insertion of a needle beneath the skin), suffering, or distress. These individual procedures will not result in the significant impairment of the well-being or general condition of the mice.

Mice that undergo surgical procedures will experience short-lived (i.e., less than 24 hours) post- operative pain and discomfort. These impacts are not expected to last for more than 24 hours.

Mice undergoing terminal procedures will be completed under general anaesthesia. This will keep the mice in a state of sleep/unconsciousness for the length of the procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse: Subthreshold 98%

Mouse: Mild 1%

Mouse: Moderate 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?

Mice are widely used in pre-clinical research. This is because their genes, biology, and behaviour are similar to humans. We can then use mice to study many of the causes and effects of disease or injury seen in human patients. Some things we can study are tissue damage and the presence of inflammatory immune cells. These are types of cells that release chemicals and molecules that prevent the damaged area from being fixed. We also use genetically modified mice. These mice allow us to turn on or turn off specific genes that are involved in the injury or disease. We can then study proteins and cellular activity and how they work in the injured or diseased brain.

Which non-animal alternatives did you consider for use in this project?

Over the years our group has refined and improved our use of cells grown and maintained in plastic dishes. Using these cells, we have exposed them to experimental treatments to understand if they have a positive or negative response. This way we can test their safety before then testing them in a mouse.

Additionally, we have developed a new way to grow and maintain human cells in plastic dishes that does not involve the use of mice. We can then use these cells to perform experiments that test our ideas about how human cells respond to treatments without having to use cells from mice beforehand. This new way of testing cellular responses in plastic dishes is closer to the response of these cells in a living human. This will be help us in being able to predict which aspects of the cell in humans are the most important to study using our mouse models.

Why were they not suitable?

Cells grown in plastic dishes are useful for studying some aspects of human disease. However, they do not fully capture the complex changes that happen to cells in a living mouse or human. Using mice allows us to study these complex changes in a setting that is more like the human disease or injury.
This way, we can understand the true function of these cells in their normal setting.

Cells grown in plastic dishes behave differently to those found in a living mouse or human. Cells in plastic dishes lose their diversity and the ability to communicate with other cell types.

Therefore, it is necessary to use mice to assess the complex biological and behavioural responses of cells following an injury or disease. This is also important when testing a potential therapeutic treatment. There is also a requirement to demonstrate that a treatment is safe and effective in animals before progressing to human application.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Mouse numbers were estimated based on a combination of the retrospective review, annual return of procedures, and the estimated animal usage needed to supply the experimental project licenses with our group for their duration.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs) website provides excellent resources for implementing colony management best practices. This includes important considerations to create suitable breeding strategies to reduce the production of mice not carrying the genetic modification of interest for use on other projects. This resource is used as a reference to design breeding strategies prior to the mating of genetically modified animals. This will avoid the excessive waste of mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding of mice colonies will be planned with care to avoid the creation of surplus stock by carefully and consistently tracking the number of mice.

Any line of genetically modified mice not actively being used in our experimental work will be removed from active breeding and the line frozen down to prevent the generation of surplus stock.

Breeding mice will be replaced before their reproductive performance declines. This will be done by maintaining breeder mice of various ages by replacing a percentage of them monthly.

Non-productive breeders, i.e. those female mice that have not produced a litter within 60 days of mating or since their last litter, will be replaced. Breeding mice will be young, sexually mature male and female mice as younger mice generally breed better than older ones.

Where possible, experienced males will be housed with size matched young females to improve breeding performance and prevent injury to the female mice during mating. Additionally, to produce offspring with the same age we will house females together in

pairs to sync up their reproductive cycles. Then, these female mice will be housed with individual males which will result in the maximum number of pregnancies.

We will keep meticulous and accurate breeding records to evaluate the breeding performance of the mouse colony. This will allow us to detect problems sooner so that they can be corrected.

We are also coordinating with other groups to share animal tissues – including tissues from genetically modified mouse lines and post-mortem tissues - in order to further reduce overall animal numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using a non-surgical method to increase the number of viable embryos that are released from female mice to be available for fertilization.

We will be using a surgical method under anaesthesia in which mouse embryos are placed into the uterus of a female to establish a pregnancy.

We will use a non-surgical method in which mouse embryos are placed into the uterus of a female to establish a pregnancy.

We will be using a surgical method under anaesthesia in which male mice will undergo a vasectomy to render them sterile.

We will be mating and breeding male and female mice to produce offspring for use in our experimental licenses.

These methods are standard in the breeding and maintenance of mice for scientific purposes and have been routinely updated and optimised to cause the least pain, suffering, distress, and lasting harm to the mice.

Why can’t you use animals that are less sentient?

We are extremely limited in the use of non-mammalian species (i.e., invertebrates (e.g., worms), fish, or amphibia). These non-mammalian species are not fully suitable for the study, development, and testing of treatments for use in humans (i.e., mammals). In fact, while some work is done in non-mammalian species, the complexity of the interactions in an organism can only be studied in mammals (e.g., mice). This is because they have similarities in organ, tissue, and cell structure with humans.

We need mice with cells that have reached a mature stage of development. This is to

make sure it is similar to the cells present in adult humans. The use of mice during the beginning stages of life will be restricted to the collection of cells to grow and maintain on plastic dishes to perform experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Female breeders will only be allowed to have six litters of newborn mice or only breed for six months, whichever comes first, before she is retired.

Stock mice that are required to maintain a live colony will be kept to a minimum to ensure appropriate mouse numbers for experimental purposes. This will avoid producing more mice than is necessary and reduce the wastage of mice.

If a strain of genetically modified mice will not be used for research purposes in the next six months, it will be archived through cryopreservation of female embryos and/or male sperm. This will preserve the genetic background of the strain until it is again needed for use in our experimental licenses.
Cryopreservation involves the collection of non-implanted embryos from female mice and/or sperm from male mice followed by long-term storage in extremely cold temperatures (i.e., -80^{o}C). This saves significant space and mice care resources. Ultimately, it allows us to better manage our colonies being actively used to supply our experimental licenses.

We will select the most appropriate breeding strategy on a per strain basis. For example, if a manuscript is under review, and a colony is being maintained in the event that reviewers request further experiments, an intermittent breeding strategy will be used to avoid animals being wasted. This is where we would reduce the number of active breeding pairs. For example, if we needed 6 breeding pairs to supply our experiments, we would reduce this number to 2 breeding pairs while the research is under review.

Our breeding strategy for mice with genetic modifications will be to set up male and female mice either in pairs (i.e., one male and one female) or in trios (i.e., one male and two females. The use of trios increased breeding efficiency to produce the mice with the desired genetic modification. A well- designed breeding strategy will lead to the largest number of offspring with the expected genetic modification. This will reduce the wastage of mice that do not have the genetic modification.

We have also designed our breeding strategy to produce offspring that either have the genetic modification or do not have the genetic modification. When these mice are supplied to our experimental licenses, they allow us to determine if the changes we have made are real.

Female mice with offspring, called pups, will be provided soft nesting material to keep the pups warm and protected. As the mice develop and approach the weaning stage (i.e., the removal of the offspring from the mother), wet mash will be provided to ensure the pups do not suffer from malnutrition.

Mice undergoing surgical procedures will be administered pain medication immediately before and after the procedure. Mice will be kept under anaesthesia during the entirety of the procedure. They will also be placed on a heating pad to maintain normal body temperature for the entirety of the procedure. Following all surgical procedures, the mice

will be placed into individual cages with a soft, grippable bedding to allow ease of movement. The mice will also be provided with access to a diet gel to maintain hydration. The cages will be kept in a heated chamber to maintain a stable body temperature. Mice will be monitored until they are awake, alert, and active (i.e., moving freely in the cage) and then returned to their home cage. Mice will be monitored daily for 7 days to ensure the surgical site remains closed, there are no development of clinical signs of distress and suffering, and to minimise the chance of post-surgical complications. Pain medications will be provided as needed to mice through either intraperitoneal (e.g., into the abdomen) injections or as a palatable substance (e.g., in the drinking water).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidance provided in the ‘Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes’ and the Jackson Laboratory. We will also use the resources available for colony management and breeding strategies on the ‘National Centre for the Replacement Refinement & Reduction of Animals in Research’ and ‘Laboratory Animal Science Association’ websites.

In addition, we will refer to the Jackson Laboratory resource manual on ‘Breeding Strategies for Maintaining Colonies of Laboratory Mice’ as well as the many manuals and guides available on their website for continued guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The National Centre for the 3Rs (NC3Rs) will be the main reference to understand whether our experiments match the highest standards of 3Rs. We will adapt our protocols if the recommendations evolve throughout the duration of this project. Regular consultations on the latest practical guidance from Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) will provide additional sources of new recommendations and advances in animal techniques and clinically applicable models.

As a license holder, it is my own responsibility to stay updated on published best practices. I will do this by consulting information for license-holders provided by our establishment and by speaking to other project license holders.

72. Anticancer Drug Discovery, Target Validation and Experimental Therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, 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 aim of the project is to identify and validate new molecular targets and pathways which cancers rely on for survival and growth and then to discover drugs or new combinations of existing drugs, which exploit the targets and translate this into new treatments for cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is now the major cause of death from disease in all age groups in the United Kingdom with approximately 300,000 new cases and 150,000 deaths each year. The

lifetime risk of developing cancer is currently 1:3 and this is expected to rise towards 1:2 as life expectancies increase. Despite improvements in early diagnosis, the majority of patients present with disease which has spread from the original site to other areas of the body (metastatic disease) and hence cannot be cured by surgery and local radiotherapy. Drug therapy (chemotherapy) is currently the only modality with curative activity against metastatic disease, notably certain childhood tumours, haematological malignancies and rare adult cancers, and it can also significantly improve quality of life of patient where cure is not possible (palliative effects) in multiple common tumour types. Nevertheless, currently available drugs do not cure the majority of patients and there is an urgent need to develop new chemotherapies with significantly greater activity than those available. The current application seeks to allow the identification of new cancer drugs.

What outputs do you think you will see at the end of this project?

The expected benefit is the identification of new drugs for the treatment of human cancer, where there is a high unmet need for new therapies. The studies will not only help to select agents for clinical evaluation, but will also inform on their optimal use. In addition to the potential therapeutic value of these agents, the studies will also provide data that will inform basic research into the targets of the agents studied.

Who or what will benefit from these outputs, and how?

In the short term our target identification and validation studies will produce data supporting or rejecting the particular target which will help the cancer research community to exclude targets which will not produce anti-tumour effect and give, well validated, new potential areas of research to concentrate on.

Early drug discovery may identify tool compounds which will help the field further investigate the pathways involved in cancer development examples of this are CDK2 inhibitors which have been widely used by many groups in the field.

In the longer term, which may be beyond the life of this licence (for example PARP inhibitors took 10 year to go from the first preclinical studies with the early lead to the first clinical trial), we would hope to bring new drugs to the clinical which will directly benefit patients.

How will you look to maximise the outputs of this work?

We have entered into a strategic alliance with an industrial partner in order to optimise our drug discovery processes. This involves our partner being briefed on our target validation studies at the earliest stages of in vitro investigations.

This allows us to not only get independent scientific feedback from their scientific advisory board on our target strategies but also access to their resource and drug discovery expertise on projects they wish to take forward with us.

We endeavour to publish all our findings both successful and unsuccessful in high quality journals and present at both national and international meetings. Publication on successful project can sometimes be delayed due to patent protection but such studies are published as soon as commercial considerations have been addressed.

Species and numbers of animals expected to be used

- Mice: 9800
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The majority of animals used will be adult mice although rats will be used where there is scientific rational suggesting rats better reflect humans or for practical reasons when the size of rats make them a better model.

Typically animals will be immune compromised to allow the growth of human tumour xenografts (tumours formed from human cells in an animals models, literally meaning foreign graft, or biological material in the form of cells, organs or fragments of tissue grafted from one species to another) in order to study the pharmacology (the study of drugs and how they work) of cancer treatments and discover new treatments.

Where mice are genetically altered (other than immune compromised) this is usually in order to better understand human diseases and seek new treatments.

Wild type mice may also be used to assess toxicity or be implanted with syngeniac (from the same species eg mouse tumour grown in mice) tumours to study the involvement of immune response in cancer treatment.

Typically, what will be done to an animal used in your project?

Typically mice on most of the protocols will be used to assess well new drug treatments or combinations of treatments work or the pharmacology (pharmacokinetics (how the body deals with the drug in terms of absorption from the gut, distribution around the body and elimination (excretion) and pharmacodynamics (how the drug effects the body in terms of desired effects (efficacy) and undesirable or side effects (toxicity)) of these treatments in mice bearing tumours.

In order to do this it is essential that animals develop tumours. Tumours may occur naturally due to an inherited abnormality (<4%) or may be induced by implantation of tumour cells either under the skin (sub cutaneous ~76%), intravenously (by injection into a vein <4%) or surgically (~16 %) into and specific site such as the liver (<4%), bone marrow (leukaemia) (<4%), pancreas (<4%) or bladder (<4%).
Growth and development of tumours may be monitored using medical imaging techniques including but not exclusively MRI, CT ultra sound etc.

In order to aid the development of tumours some animals may receive whole body, irradiation e.g. treatment with x-rays(<5%).

Some animals may also undergo castration (<5%) or ovariectomy (>5%).

In total these studies would account for approximately 85% of all the animals used.

The remaining 15 % would be used in pharmacokinetic or toxicity studies and would be split into:-

wild type mice or rats used in toxicity studies in MTD studies of new pharmacophores (the core chemical structure around which new drugs are built) (~3% of total)

or mice which are wild type (normal) and genetically modified (GM) (GM mice have been modified to have an alteration to their genetic make up which makes them susceptible to a particular cancer, an example of this is the breast and ovarian cancer related gene BRCA in humans) paired where studies may be performed to check the involvement of the target in any toxicity (side effects) observed (~2% of total).

or wild type mice or rats used in pharmacokinetic studies to determine biodistribution (how the drug moves around the body to different organs) bioavailability (how much of the drug actually gets into the body after an oral dose) and metabolism (how the body breaks down the drug) and elimination (how the drug is excreted) (~8 % of total).

or mice which are wild type and GM paired where studies may be performed study the pharmacodynamics and develop specific biomarkers for target engagement (~2% of total).

Imaging techniques may be used to follow the pharmacokinetics of drugs over a 8 hour period in a single animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Generally animals may show signs of sickness e.g. hunched posture, diarrhoea, ruffled fur, look pale or feel cold. Supportive care and pain relief will be given and if the clinical condition does not improve within 24h mice will be humanely killed. Tumour models: Sub cutaneous models where tumour grow under the skin may cause minor discomfort. they may grow upto ~1.5 cm in diameter but will not be allowed to break through the skin.

Orthotopic models where tumours are grown in the organ in which they would naturally occur (all of the surgical models:- liver, leukaemia in bone marrow, bladder and pancreas) and naturally occurring spontaneous tumours due to an inherited abnormality would be expected to show adverse effect associated with the site of implant eg jaundice for liver tumours, careful monitoring of tumour growth will ensure such events are rare however, if such effect are observed then additional supportive care and pain relief will be given and if the clinical condition does not improve within 24h mice will be humanely killed.

Surgical models: adverse effects due to surgical complications may include bleeding or inadequate anaesthesia. Supportive care such as fluids, soaked diet and a warm environment will be provided. Pain relief is always given in surgical models.

Mice may be sick and lose weight after irradiation (e.g. treatment with x-rays) but will recover once the immune system has been replaced.

Imaging: mice may experience a small weight loss post imaging but this should recover within a few days.

Therapy, we do not anticipate therapies will cause significant adverse effects. In all models pain relief is given when needed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity over 5 years

Moderate < 8800 ~ 89%, < 450 rats ~90 %

Mild approximately somewhere between 900-950, 9-16 %, ~45 rats ~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?

Use of animals is needed to address the issue of the therapeutic index of a new agent (i.e. the amount of a medicine that gives the desired effect compared to those which cause un wanted (toxic) effects. In addition the effects of medicines on the whole body both in terms of how the body takes in the medicine, how the medicine moves around the body and how the body get rid of the medicine (pharmacokinetics) and what the medicine does both to the tumour, which may be affected by the site of the tumour and the cells which make up the tumour, but also the normal tissues (pharmacodynamics) cannot be predicted in human tissue/cells or cell culture systems (in vitro models) because of the complex nature of the way the body's systems work together.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, we will use human tissue/cells or cell culture systems to replace animal models of organ fibrosis and cancer. The group have accumulated archival tissue banks of frozen and formalin fixed tissues from our previous models and human normal and diseased tissues. These samples are used in multiple on going projects to minimise the number of animal disease models used. We routinely use human cells in culture (including cell lines) to understand and model the biological processes involved in causing tissue fibrosis and cancer or to perform drug testing.

We currently collaborate with a commercial company, where compounds are screened, in order to get in vitro predictions of pharmacokinetics such as bioavailability and metabolic stability and some common toxic liabilities (eg HERG inhibition). All compounds are screened in house for their activity against their target and where possible screened against related targets either in house or more commonly by a commercial screening company.

Why were they not suitable?

Whilst these are useful tools, there are limitations of cell cultures systems, these include;

Cells grown in petri-dishes sit on plastic, which change their behaviour and they become "super sensitive" or fail to do the job they would in the body. These abnormal behaviours could lead to the identification of non-relevant pathways or fail to predict identify drugs which are likely to be ineffective in the disease.

The in vitro screen and systems we use prior to animal studies are extremely useful for aiding our basic knowledge and for initial drug screening, and we use these systems to reduce animal use however they cannot fully recapitulate the behaviour of a drug in a complex biological system..

Reduction

How have you estimated the numbers of animals you will use?

We have performed audits of our previous research studies and assessed research plans of current projects and proposed new targets to predict use under this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Only after the above rigorous in vitro development and selection experiments are animal experiments considered, and animals are never used as a random screen. In addition, all experiments are designed to use the minimum number of animals to generate a statistically valid result.

The development of the new imaging based methods may allow real time monitoring of drug levels and drug action in animals reducing the number of animals required to assess the pharmacology of some agents. In addition we have entered a collaboration to help develop in vitro pharmacokinetic prediction methods. Use of these in vitro and in silico models will further reduce the number of compounds for which full pharmacokinetic evaluation in vivo is required.

Following consultation we have modified our toxicity studies to reduce the overall number of animals used by avoiding pure toxicity studies in non-tumour bearing mice where possible. This has worked well, and has led to no increase in adverse events over those that were observed prior to this change.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Statistical analysis is performed to determine the minimum numbers of animals need to generate biologically meaningful data. With pilot studies performed, if this is not possible, to reduce numbers and inform future studies going forward.

Tissue is regularly shared between projects to maximise outputs from animal procedures and minimise numbers 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 use rodent models that have been consistently shown to reflect human toxicity (side effects) and provide a robust background for the development of new therapies in cancer research. The majority of studies being in mice with rats used where either their larger size is necessary for practical reasons or where the pharmacology of the drugs to be studied has been found to be better reflected by rats physiology in the in vitro studies which precede in vivo studies.

The majority of anti-tumour of tumour pharmacokinetic/pharmacodynamics studies will be carried out in immune deficient mice bearing subcutaneous (sub.cut.) human tumour xenografts. Although not without its weaknesses, this model has be shown to be robust in its ability to produce good reliable data both in terms of antitumour activity and modulation of tumour biomarkers. In addition recent studies at our establishment have demonstrated that this is a relatively mild procedure producing no real evidence of pain in mice.

Orthotopic models (models in which the tumour are grown in the organ of origin eg pancreatic tumours grown in the pancreas) can address some to the challenges associated with the tissues and organ structure in the areas of the tumour (tumour microenvironment) and the ability to get medicines to the tumour in these places (tumour drug disposition). However, whilst studies in bladder and intrafemoral tumours have shown these also cause little evidence of pain behaviour in mice (at least until tumour burden becomes such that it interferes with normal physiological processes) tumour in other sites may cause pain and discomfort. It is therefore important that the growth of such tumours is carefully assessed and characterised in order to reduce potential harms to the absolute minimum. In order to do this and to quantify the growth of orthotopic tumours imaging techniques are required, for example CT or ultra sound, these technique require the use of repeated general anaesthesia to immobilise the animals. whilst these imaging technique will help reduce potential harms from the tumour and its growth, general anaesthesia is not without its own risk of adverse effects. For these reason orthotopic tumours will only be used if there is strong evidence that a new therapy may have a potential role in the tumour type, for example if the drug target is over expressed in the particular tumour type. Even in these cases it would be normal practice to test the drug in a subcutaneous tumour model (where the tumour is grown under the skin) first.

Why can’t you use animals that are less sentient?

Many groups now use the zebrafish model to investigate the mechanisms of oncogenesis (the process of normal cells transforming into cancer cells). Whilst this is a useful model and can provide an insight into the development of cancer and help identify targets that may be used in drug discovery it is not an appropriate model for our drug discovery research. In order to exploit targets for clinical development we need to study human cancers with a well-defined molecular- and histo-pathology, first in vitro and then in in vivo

in a mammalian setting, where we can interrogate drug exposure, tolerability and efficacy.

Zebrafish embryo's are also used to look at growth of human tumours however these are short term models as the embryo's develop immunity. These models may be useful in showing is a gene or target pathway drives a cancer in a way similar our xenograft studies but we would then still have to validate the model to use in later studies thus no reduction/refinement would result. Zebrafish embryo's have also been used to screen drugs however like in vitro studies these studies don't encapsulate the complexities of the mammalian system. Development of the embryo further complicates the picture in terms of looking at the long term effects of tumour which may appear to have been cured. So whilst these models may be useful to run along side studies in mammals they cannot generate the data we would need to take our drugs forward

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals, regardless of disease model are checked regularly and supportive care is readily provided to minimise distress or suffering and improve animal welfare.

For surgical models we use good surgical techniques and operating theatres/equipment to minimise the risk of infection. Mice receive pain relief and a high level of post-operative care including soaked diet, a warm environment and fluids as required to minimize stress and suffering.

Our procedures are designed with animal welfare at the forefront to ensure the minimum suffering and distress to the animal to which end we are collaborating with the animal welfare group in order to assess any pain caused by experimental tumours and facilitate the appropriate use of analgesia. We have also actively engaged in a program to assess how tube handling can reduce the stress in experimental models, which has now been adopted as standard practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all models and optional procedures good practice guides will be used to help refine the model as described in the ARRIVE guidelines and Laboratory animals special article 2015, 49 (s1).

LASA Working Party guidelines on assessment and control of severity will be used throughout the project to determine if any animal is suffering distress.

Workman et al in 2010. British Journal of Cancer (2010) 102, 1555–1577 NCRI guidelines will be used to perform and monitor cancer studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are many sources in which provide information regarding 3Rs advances, these include; the NC3Rs website, NC3Rs seminars/events and emails, scientific publications and published guidelines as well as continued professional development e.g. local seminars, regular communication with the NACWO and veterinary team and academic collaboration with the welfare group.

As information on welfare or technical improvements, alternative less severe models or new non-animal model systems becomes available an appropriate strategy within the research group and veterinary teams will be implemented to ensure that animal use and suffering is minimised. This will include testing new models (animal or non-animal) and modifying procedures.

73. In Utero Therapy for Congenital Blood 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

In utero Therapy, In Utero T Reg Therapy, Stem Cell Transplantation

Animal types	Life stages
Mice	embryo, neonate, pregnant, 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 Treat Congenital Blood Disorders before Birth (prenatally) using cell therapy

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diseases inherited through families, such as those affecting the genes in red blood cells, e.g. sickle cell disease (SCD), are not very common but can severely affect lifelong health. I am working to see whether correcting the faulty red blood cell gene (sickle cell gene) in the developing baby whilst still in the womb could correct or reduce the problem before the baby is born. Approximately 400,000 births per year are affected by SCD. It is inherited as an autosomal recessive condition caused by haemoglobin beta-globin gene mutation.

Without appropriate treatment, SCD is often fatal in childhood. In low to middle-income countries where 90% of SCD cases occur, an estimated 50 to 90% of children born with the condition are dying within or not reaching their 5th birthday. This project will address the need to promote the correction of SCD before birth. We will use advanced gene medicine with nanoparticles or genetic vehicles to target the defective blood-forming cells in the fetus before they begin their final journey to the bone marrow. We will assess the safety and feasibility of this approach using a well-described mouse model of SCD. In addition, we will test the gene medicine on human cells collected from the cord of SCD at birth.

What outputs do you think you will see at the end of this project?

Diseases which are inherited through families, such as those affecting the genes in red blood cells e.g. sickle cell disease, are not very common but can have a serious effect on lifelong health. We are working to see whether correction of the faulty red blood cell gene (sickle cell gene) in the developing baby whilst still in the womb could correct or reduce the problem before the baby is born. In this project, we will aim to "reprogram" the early fetus by using new technology which will allow the recognition of the donor cells with the corrected genes as 'self'.

This project will address the need to maximise the efficacy of stem cell (HSC) transplantation and to promote the correction of gene disorders in utero. Currently, researchers in the USA are conducting an in-utero stem cell transplantation trial using maternal stem cells transplanted into the fetus. This involves maternal stem cell mobilisation, isolation and fetal transplantation, which comes with risks to the mother herself. Suppose the mother has sickle cell genes on both chromosomes in her DNA, for example. In that case, this also requires correction of the gene defect in the laboratory using gene editing approaches, which makes the process complicated and very expensive. Our project is novel in addressing the role of immune cells known as Tregs in helping the fetus accept the HSC containing the corrected gene. It will complement and enhance the current local transplantation research programme in defining the contribution of these cells to facilitating the survival of fetuses given the HSC. This has the dual purpose of targeting a niche not currently being studied by the global scientific community. In addition, using a clinically relevant sickle cell disease model meets the translational challenge of promoting the correction of single gene defects prenatally.

We work especially closely with the basic science focused Treg biologists and the clinical maternity services, although ultimately, we envisage that our findings will augment other research groups' activities through the provision of specific immunology expertise focused on tissue regeneration, samples and models and sharing of high-level scientific technology.

Prenatal diagnosis is available across the UK and worldwide. Currently, parents have only two choices when faced with a prenatal diagnosis of sickle cell or thalassemia, either to accept an affected baby or termination of pregnancy. This treatment will be offered as a third option to the couple who currently have a difficult decision, termination or continuing with the pregnancy and, if available, postnatal treatment.

If successful, treating an affected fetus would avoid the need for postnatal treatment. This would have a huge impact, especially in those countries such as Africa, the Middle East and the Far East, where termination of pregnancy may not be available, blood transfusions are prohibitively expensive, and where blood disorders are most prevalent.

Who or what will benefit from these outputs, and how?

Impact on the Society

- Offer the option of prenatal treatment to parents.
- Reduce pregnancy terminations
- Reduce the enormous economic impact, which an illness like Sickle Cell has on health systems. In England alone, between 2010- 2011, 6077 admissions were recorded associated with sickle cell disease. The total cost for these admissions was £18798255-the price increases with age, with children staying longer than adults.
- Off-the-shelf treatment, to be made available in low resource settings

Impact on the Scientific community

- Scientists can use the pre and post-gene editing sequencing datasets for further analysis and compare the efficiency of corrections with their research.
- Repeat the experiments by following the detailed experimental design and data, which will be made available via an open-source data archival system.
- Bioengineers working on fetoscopic therapy tools can better understand the size of the delivered gene-editing tools to develop appropriate fetoscopic instruments for human use.

How will you look to maximise the outputs of this work?

The findings will be disseminated at national and international meetings such as The European Society of Gene and Cell Therapy, the American Society of Gene and Cell Therapy (ASGCT), and the International Society for Stem Cell Research (ISSCR). We hope to publish our findings in high impact journals such as Blood and Molecular Therapy. We will also be involved in the Sickle Cell Society and organise public engagement events, with the involvement of the MRC, to disseminate the results to patients and relatives. We will be participating in various fundraising events, such as the Prudential Ride, the London marathon, allowing us to publicise our research.

We aim to present our findings at national and international meetings such as The European Society of Gene and Cell Therapy (ESGCT), the American Society of Gene and Cell Therapy (ASGCT) and the International Society for Stem Cell Research (ISSCR).

We aim to interact with people who are affected by the disease and obtain their thoughts, on the future presence of definitive treatment (Sickle Cell Society UK). This will allow us to understand whether patients would be willing to allow clinicians to deliver stem cell therapy in utero instead of terminating the pregnancy or wait for postnatal management. By establishing patient support, this will also increase the popularity of the study among scientists and sponsors who will be interested in getting more actively involved.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using these animal models because they recapitulate human disease (Sickle Cell Disease). We will be injecting these animal's fetuses at E13.5-E14 because at that point the main haematopoietic organ is the liver which contains most haematopoietic progenitors prior to bone marrow migration. This will maximize therapeutic engraftment.

we are using the FoxP3 mouse where enhanced green fluorescent protein is expressed in FoxP3+ T cells. These mice may are useful in our proposed studies of regulatory T cell function in the induction of tolerance.

Typically, what will be done to an animal used in your project?

All the experimental mice will be housed in a single cage after plugging.

The pregnant mouse mothers will be anesthetised and dressed using sterile drapes. The skin hair of the mouse will be removed, and the abdomen will be disinfected.
A midline laparotomy incision will be carried out to expose the pelvic cavity and the pregnant uterus. The liver and vitelline vein of each embryo will be identified. A total of 10-20µl of the solution containing the prepared cells/gene vector will be delivered either intravenously, intraperitoneally, or intra-cardiac using a 34 gauge needle attached to a microinjector system.

The uterus will be kept wet in warm sterile normal saline. The uterus will be repositioned in the abdomen. The abdomen will be closed with continuous suturing for the peritoneum and subcuticular suture. A local anaesthetic will be infiltrated around the incision.

The dams will be transferred to a warm cage for initial recovery and then to a regular cage. The dams will be checked daily and given wet food.

The transplanted pups, when born, will be cross-fostered to time-mated CD1 dams on Day 1 to avoid maternal cannibalism and antibody response from the mother. The mother will be killed using a schedule 1 method after cross-fostering.

The recipients will go under venepuncture for engraftment analysis at specific time points. Postnatal tail injection of stem cells or substances to boost the therapeutic effect might be needed in some recipients.

What are the expected impacts and/or adverse effects for the animals during your project?

Anaesthesia

For general anaesthesia, the appropriate depth of anaesthesia often depends upon the strain. Therefore, under- or over-anaesthesia will be minimised/avoided by continual monitoring of breathing rate. Adult mice will only receive inhalation anaesthesia, usually isoflurane. Injectable anaesthesia will be used only very rarely.

Hypothermia is a common adverse effect of general anaesthesia for surgery in rodents.

Therefore all anaesthesia will be performed while keeping the animal on a thermally-regulated pad or in a thermally- regulated chamber. After anaesthesia, animals will be observed until they are awake, in a heated recovery box before being returned to their cage to prevent such an effect.

Surgery

The animal will be monitored continuously until it has fully recovered. They will be allowed to recover in a warmed chamber not exceeding 32°C. Possible pain during/after surgery will be minimised using appropriate analgesia, as recommended by the NVS or NACWO. Where signs of distress are observed (lack of mobility, discomfort, hunching or piloerection) via video/physical observation post-surgery, analgesia will be administered as required. Animals may show signs of distress immediately post- surgery; they will be assessed closely one hour after surgery, at the end of the day, and the following day. If they are still showing signs of distress, they will be culled.

Complications, including infection and wound dehiscence, may rarely occur due to surgical manipulations. This will be minimised by the use of the appropriate aseptic technique. After the procedure, frequent observation will be undertaken to ensure that wound healing and recovery are free from bleeding and infection. In the unlikely event of wound breakdown, the animal will be culled using a schedule 1 method. Any evidence of bleeding during surgery will necessitate gentle compression until bleeding is stopped. In the rare event of bleeding from the wound or signs of infection, animals will be removed and killed under Schedule 1

Surgery for in-utero therapy may result in the maternal cannibalisation of newborn pups. This varies from strain to strain and between animal houses. Strains will be crossed onto the CD1 background where cannibalisation is uncommon (<5% of litters). In addition, the NVS/NACWO will be consulted for possible husbandry strategies to improve mothering e.g. co-caging of gestation-matched experimental and CD1 pregnant dams. As a last option, a caesarean section of the mother and cross-fostering of the pups may be performed.
Vector-related adverse effects

The vectors under investigation are not known to induce any adverse side effects. However, overexpression or ectopic expression of a transgene may, hypothetically, induce unpredictable adverse effects. If these are observed, subsequent experiments will use a diluted vector, dropping 3-fold or more at each round.

Expected severity categories and the 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 only strain experiencing moderate/severe phenotype is the Sickle cell model. Mice homozygous for the alpha-globin null allele, homozygous for the beta-globin null allele and carrying the sickle transgene (Hba0/0 Hbb0/0 Tg(Hu-miniLCRα1GγAγδβS) are called sickle cell mice (Berkeley model).
They exclusively express human sickle hemoglobin, and do not express mouse Hba or Hbb. Although chronically anemic, most of these mice survive for 2 to 9 months and are fertile. A significant percentage of sickle cell mice do not survive to adulthood. These mice

display the major genetic, hematologic and histopathologic features observed in humans with sickle cell anemia; including irreversibly sickled red blood cells, anemia and multiorgan pathology. Typically, ~20% of sickling mutant mice die between weaning and 14 weeks of age.

In the event that signs of infections occur (such as ≥15% body weight loss, lethargy, piloerection, hunched posture, difficulty moving, pallor), mice will be euthanized using a schedule 1 method.

Infection should though be a rare occurrence.

Any bleeding after blood sampling vial peripheral tail vein bleeds will be controlled by the application of local pressure.

Blood sampling will not exceed 15% total blood volume in any 4-week period. Adverse effects of blood sampling are however unlikely.

Adverse effects of the injection are unlikely, but there may be mild discomfort at the site of injection.

In cases of significant inflammation, ulceration or swelling sufficient to cause distress, animals will be killed by a Schedule 1 procedure.

Agents transferred to promote engraftment:

No specific side effects of immunosuppressive agents, cytokines or antibodies are anticipated. Mice will be routinely assessed for signs of ill health, such as weight loss greater than ≥15% of body weight, diarrhea, isolation, hunched appearance, piloerection etc, and any animal displaying any of these signs will be killed using a Schedule 1 method Immunosuppressive protocols. The immunosuppressive protocols reduce but do not abolish immune responses.

The animals' water consumption and hydration status will be monitored daily until it has been established that the animals are hydrating well.

Topical antibiotic cream with or without systemic antibiotics will be applied in such instances to treat the infection. Any animal that develops ulceration or skin infections that cannot be effectively treated within three days, or is in distress, will be killed using a schedule 1 method.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We will use in vitro assays as much as possible to replace in vivo experiments. Initially, in

vitro and ex vivo assays may provide insight into the cellular mechanisms involved in the induction of tolerance and allograft rejection. However, they cannot adequately model the complete array of important immunological factors and cells in vivo. Therefore, further in-vivo work is required.

In vivo studies also avoid artefacts that can occur when immune cells are removed from their environment and subject to strenuous purification procedures.

Which non-animal alternatives did you consider for use in this project?

Alternative techniques that could replace the use of animals in our in vivo experiments can be applied such as in vitro assays. This has already been accounted for, and the minimum number of animals will be used in this section. Unfortunately, we cannot replicate the in vivo system, so the final experiments are needed for confirmation.

In the case of in utero transplantation, unfortunately, this requires in vivo testing.

in addition, we aim to collaborate with the researcher who designs the “Virtual Assay” software developed in a research institution. This uses computer models based on human data, which potentially could be a more effective alternative to some of our in vivo experiments, probably the postnatal stem cell transplant study.

Why were they not suitable?

As explained above, unfortunately, we cannot replicate the in vivo system, so the final experiments are needed for confirmation after in vitro testing.

In the case of the in utero transplantation, unfortunately, this requires in vivo testing.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

As noted above, we will endeavour to use in vitro assays wherever possible thus limiting the numbers of mice undergoing experimental procedures.

Although experimental mice will not be used on multiple protocols (other than breeding), to reduce mice usage, donor mice may be used for multiple experiments. For example, a mouse culled by schedule 1 that is used to collect donor skin, may also be used as a cell donor, or spleens and bone marrow for in vitro assays.

The sizes of experimental groups and the number of repeated experiments will be kept to a minimum while ensuring that reproducible results are obtained with clear biological significance. When preliminary data is available, power analyses will be used to determine the minimum numbers of animals and repeated experiments that are required to meet statistical significance. The number of mice that are required varies depending on the

model used.

I have been carrying out murine in utero transplantation since 2012 I have, in collaboration with the NVS, refined these techniques to minimize the numbers of mice lost due to surgical errors.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have been carrying out murine in utero transplantation since 2012. We have, in collaboration with the NVS, refined these techniques to minimise the numbers of mice lost due to surgical errors.

Prior to any animal testing the NC3rs experimental design assistant https://www.nc3rs.org.uk/experimental-design-assistant-eda will be used to reduce animal numbers.

Breeding of the transgenic colonies will be done in collaboration with the BSU and a minimal number of breeding pairs will be kept to avoid unnecessary culling of unwanted animals. In addition, we will use a commercial genotyping service to avoid any delays in identifying homozygote sickle cell animals and wild types.

We are planning to publish our results according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
Before the proposed study, a feasibility study (n=3), will be done to test logistics and gather information prior to proposed for larger study, to improve the latter's quality and efficiency.

This will be used to reveal deficiencies in the design of a proposed experiment or procedure and these can then be addressed before animals, time and resources are expended on large-scale studies.

Before conducting the pilot study, a systematic review of the literature will be done to fine-tune the number of cells and techniques before animal testing.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen mice, as we are principally studying models of inherited genetic blood disease and mouse models are available for most of them. In addition, mice have an immune system of comparable complexity to human. For these reasons, mice are the

most frequently used animals in studies of the human immune system.

Where possible, we attempt to prevent the onset of the disease, rather than treat it once the disease phenotype has been displayed; this prevents undue suffering.

We will use a sickle cell mice, which exclusively express human sickle haemoglobin and do not express mouse Hba or Hbb. Although chronically anaemic, most of these mice survive for 2 to 9 months and are fertile. A significant percentage of sickle cell mice do not survive until adulthood. These mice display the major genetic, hematologic and histopathologic features observed in humans with sickle cell anaemia, including irreversibly sickled red blood cells, anaemia and multiorgan pathology. Typically, ~20% of sickling mutant mice die between weaning and 14 weeks of age.

This project aims to breed transgenic mice for use in biomedical research. These animals are needed to investigate the role of different immune cells and the influence of genetic factors in a transplantation setting. The mouse models are important, not only in providing new insights into the processes that lead to rejection but also as models in which to study novel transplantation therapies.

All the protocols proposed in this application utilise well-established and tried techniques that have been refined to involve minimal suffering. The most invasive procedures will involve in utero stem cell transplantation and gene therapy. Anaesthesia and analgesia will be administered to minimise discomfort and the animals will be assessed daily for any signs of distress. In all the proposed in vivo models, if animals display signs of distress, advice will be sought from the NVS and if distress cannot be alleviated, the animals will be culled by a Schedule 1 method.

Why can’t you use animals that are less sentient?

In protocol 1, the testing will be done using in utero transplantation in an E13.5 fetus; we expect minimal suffering of the fetus. The dam will be under general anaesthetic with local anaesthetic and painkillers mixed with the water will be used postoperatively.

How will you refine the procedures you're using to minimize the welfare costs (harms) for the animals?

We will liaise regularly with the NVS and the NACWO regarding the welfare of the experimental animals and breeders.

A systematic review of the literature will be performed before any in vivo study.

A pilot study will be performed to refine the experimental methods and reduce animal numbers and suffering.

The dams after surgery will be transferred to a warm cage (28oC) for initial recovery and then to a regular cage.

The dams will be checked daily and delivered the recipient mice 6-7 days post-IUT (E20-21). All the operated mice will be given wet food for the first day and regularly monitored for any signs of infection, especially during the first seven days.

The newborn pups will be cross-fostered and were placed in a cage with a CD1 dam, that

will be time- mated and plugged one day before the treated dam. The cross-fostering will be performed ideally at day 1 post-natally to avoid maternal cannibalism.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I am planning to follow the Prescott MJ, Lidster K (2017) Improving the quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217 and any future publications by the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stay informed by attending seminars and webinars by www.NC3rs.org.uk the IAT.

I am planning to attend the annual NC3Rs/IAT Animal Technicians' Symposium.
I received the NC3Rs newsletter

In addition, I follow the national centre for the 3Rs Twitter and regularly visit the NC3Rs website. In addition, I am in contact with the supplier, which will be providing the transgenic animals via the supplier and my local BSU.

74. Enhanced Bone Ingrowth

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

Bone, Ingrowth, Enhancement

Animal types	Life stages
Sheep	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to evaluate the safety and effectiveness of new materials and surfaces to be used in orthopaedic surgery. The primary aim of this project is to develop novel therapeutics and surgical techniques to facilitate bone ingrowth.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work to be carried out under this project licence will be done to improve the experience of patients undergoing joint replacement. It also aims to reduce costs for the Health Service due to shorter stays in hospital.
These experimental models for studying bone ingrowth need to reflect the biomechanics and the physiology of the particular clinical scenario in humans. The therapeutic materials may be either (a) synthetic, i.e. polymeric (resorbable or non-resorbable scaffolds, or a

combination of both), metallic or ceramic or biologic (growth factors, cells, signalling molecules, placental tissue and bone marrow aspirate).

What outputs do you think you will see at the end of this project?

The primary output of this project is to develop new products/methods to enhance bone ingrowth using existing experimental in-vivo models developed under previous licences. Additionally, this will help gather scientific knowledge and generate publications to better the wider scientific community.

Although, the current set of in-vivo models have shown to translate clinically, there is still an opportunity to expand and increase the clinical relevance of the models available. Therefore, a secondary objective will be to develop new experimental in-vivo models for studying bone ingrowth that better reflect the biomechanics and the physiology of the particular clinical scenario in humans. These new models will in turn, facilitate the development of innovative products, which will improve patient outcomes.

Who or what will benefit from these outputs, and how?

SHORT-TERM BENEFITS (0-3yrs): Although, the current set of in-vivo models have shown to translate clinically, there is still an opportunity to expand and increase the clinical relevance of the models available. These new models will in turn, facilitate the development of innovative products, which will improve patient outcomes. The project will attempt to develop new experimental models of bone ingrowth, which will help advance the frontiers of science in this area for the benefit of clinicians appraising preclinical bone ingrowth studies. It will also allow us to investigate new indications for some of their strategic acquisitions, which could be used to enhance bone ingrowth. These therapeutics will be tested using existing bone ingrowth models developed under previous licences.

LONG-TERM BENEFITS (5-10 years): The project will also provide long term benefits for patients undergoing joint replacement will benefit in the short-term as they should be able to return to a day-to- day life much quicker and experience less pain. Long-term benefits would be the economic benefits which include fewer days lost at work, fewer hospital days and reduced care costs.

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 ingrowth. 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

- Sheep: 400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

SHEEP: Sheep have been selected as a large animal experimental model, given that their anatomical size and skeletal dimensions is similar to humans, and ease of supply. Sheep have also been selected for blood sampling due to the volume of circulating blood available and their greater availability. Skeletally mature sheep are preferred over juvenile and aged animals in this licence for the following reasons; (a) availability from the open market, (b) our current implants and surgical instrumentation also tailored towards the bone of skeletally mature animals, (c) bone ingrowth and remodeling processes are dissimilar between juvenile and skeletally mature animals and so juvenile animals are not considered to be sufficiently representative of the clinical conditions under study. From past experiences, aged sheep are more susceptible to succumbing to complications arising from the surgical procedure and the effects of the general anesthetic. Therefore, for this reason young adults are more suitable.

Typically, what will be done to an animal used in your project?

Surgically, under general anesthesia with recovery appropriately sized defects will be drilled into suitable cancellous bone sites These defects will be filled with either test materials, industry standard controls or left as empty defects. The defects may be created in both hind limbs; up to four defects will be created per animal. The estimated time of the surgical procedure is approximately 1.5 hrs. 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 3 years later. At the end of the study, the animals will be humanely euthanized.

What are the expected impacts and/or adverse effects for the animals during your project?

The project will involve the creation of a bone defect, which may lead to some degree of discomfort following surgery, although this will be reduced by the use of a minimally invasive technique. Any discomfort will be minimised with the use of 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 up to 95% of animals will experience moderate harm in work under this protocol. The remaining 5% or less of animals will be classified as non- recovery by dying whilst still under controlled anaesthesia due to either adverse reaction to the anaesthetic itself or being euthanised due to irreparable surgical complications.

Due to the enforcement of humane endpoints in each protocol, it is highly unlikely that any animals will suffer any severe 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?

Animals will only be used where there are no alternatives to the use of animals in order to answer the questions that the project requires.

Bone ingrowth is a complex, well-orchestrated physiological process of bone formation involving signaling cascades and cellular repair mechanisms that cannot be studied by in-vitro cell culture studies alone where these complex interaction are impossible to truly replicate. Consequently, bone ingrowth can only be demonstrated with the help of animal models, i.e. no in-vitro methods such as computer simulations or cell cultures can mimic the complexity of an in-vivo environment sufficiently or predict clinical efficacy. The in-vivo models developed under this licence tailored for studying bone ingrowth will attempt to reflect the biomechanics and the physiology of the particular clinical scenario in humans.

In-vivo models will also be required when in-vitro systems cannot provide a reproducible approximation of the real-life in-vivo or clinical setting, e.g. the kinetics of delivery and distribution of drugs or bioactive factors; the biocompatibility and degradation properties of implant materials.

Initial screening and feasibility testing will be carried out in rodent models whereas large animal models, whose bone regeneration is closer to the same processes in humans, will be used to provide translational proof of concept.

Regulatory authorities such as that FDA will also require the testing of novel bone repair therapies in both a small and large animal model before accepting an agent for clinical trials to ensure clinical translation in bone tissue engineering and regenerative medicine through assessment of appropriate efficacy and safety endpoints.

Which non-animal alternatives did you consider for use in this project?

In-silico bone mechanobiology will be used where appropriate for predicting mechanobiological changes to bone tissue and investigating cell mechanobiology. In-vitro cell culture models will be used for studying bone mechanobiology in 2D by applying controlled mechanical stimuli to relevant cell lines and also to establish safety of any technology prior to any in-vivo experimentation. Collectively, these tests will be used for screening out some of the early stage technologies.

Why were they not suitable?

These preliminary tests will be used to screen out novel technologies that lack supportive data. However, in order to provide a sound basis for making determinations about reasonable safety and efficacy, animal models will be required to provide accurate information about how a medical intervention will perform in a human clinical trial. Furthermore, regulatory authorities such as the FDA and MHRA will require evidence of in-vivo safety and efficacy data prior to clinical evaluation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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.

ISO standards (ISO 10993)

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. Typically, welfare studies to assess new procedures or technologies under this licence will consist of no more than four animals. 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.

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?

COMPUTER SIMULATIONS: Computer generated models of the anatomy will be generated from CT scans of isolated limbs to create 3D models that can mimic functions of physiology and help determine safe corridors for surgical implantation. These models will also be used to develop customized implants reducing the risk of any surgical complications.

In-Vitro Testing: In-vitro cell culture models will be used for studying bone mechanobiology in 2D by applying controlled mechanical stimuli to relevant cell lines and also to establish safety of any technology prior to any in-vivo experimentation

QUALITY ANIMALS/VETERINARY CARE/PRE-SCREENING: Radiological templating of the animal prior to surgery will help screen out animals that are deemed to be unsuitable for surgery due to either health reasons or unsuitable anatomy. The images collected can also be segmented to create 3D models to assist with implant development. The loss of animals can also be minimized by providing good post-operative care, avoiding unintended breeding, and planning ahead so that the appropriate number of animals needed for the studies are ordered and/or bred.

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 either new procedures or technologies 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, histology and biomechanical testing.

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.

A thorough investigation into the most relevant species for simulating bone ingrowth have been obtained from the knowledge gained from previous project licences. Ovine geometry cointained defect and sub-articular defects are widely described and published.

Both models are already developed by our facility. These models and methods have shown that they cause the least amount of pain, suffering and distress to the animals.

HUMAN IMPLANT SCREENING: Both protocols listed in this licence will utilise sheep as this species has bones of the size that are compatible with human implants and surgical techniques. Our facility have accrued a great deal of experience with these animal species from previous licences. This experience has led to refinements in surgical technique, analgesic regimes and post-surgical care. Gait analysis has been used successfully to monitor recovery after surgery and this analysis has been used to improve post-surgical care.

Why can’t you use animals that are less sentient?

Live mammalian vertebrates are required that closely mimic the bone ingrowth pathways and human skeletal system as much as possible to ensure that any data generated can be translated to the clinical situation.

The protocols assigned to the less sentient species (rat, mice and rabbit) are generally intended to be used for the initial/earlier stage assessment of technologies tailored for enhanced bone ingrowth.
However, as the technology progresses through the product design control matrix, there will be a requirement to assess their safety and efficacy in animal species whose anatomy and physiology more closely represent the intended use in humans. These animals (sheep) tend to be of higher sentience.

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 macro- environment, (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, drug pumps).

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.

75. Cellular homeostasis and brain 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

neurodevelopment, Brain, Neurons, microglia, neurodevelopmental disorders

Animal types	Life stages
Mice	juvenile, adult, pregnant, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main aim of this project is to understand the role of pyramidal cells, the most abundant excitatory cell in the cerebral cortex, in regulating the development, distribution and maturation of microglia, the resident immune cells, in the developing cerebral cortex.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neurodevelopmental disorders such as autism spectrum disorders (ASD) affects at least 1% of the population. Currently, there are no known treatment. Furthermore, ASD are only reliably diagnosed late in development, consequently preventing earlier intervention in halting disease progression. The lack of treatment and earlier diagnosis is largely due to our lack of understanding as to how the brain develops. This work aims to remedy this by (i) increasing our understanding as to how brains are formed during development, (ii)

identification of a critical time window during development in which the brain is susceptible to perturbations and (iii) providing insights into how neurodevelopmental disorders such as ASD mayarise during development.

How does this work aim to achieve this?

Neurons are the basic working unit of the brain in which information is transmitted and processed from the periphery to the brain. Pyramidal cells, in turn are the most abundantly found excitatory neurons present in the cerebral cortex. In individuals that have been diagnosed with ASD, pyramidal cells have been known to behave aberrantly (e.g. altered levels of neuronal activity). We hypothesised that despite the different factors contributing to ASD, these factors converge onto a similar molecular mechanism, namely alteration of neuronal activity during early postnatal development. These changes can have an impact on how brains are built, especially on cell types such as the brain immune cell, microglia. Microglia are known to be involved in different aspects of brain development, more specifically in how neurons communicate with one another. Consequently, alteration in how microglia develop, mature or even the amount that are present in the brain can have a significant impact on how the brain functions. In this work, we aim to understand how changes in pyramidal cell activity can impact microglia development and how deviation from normal microglia development may contribute to some of the impairments observed in ASD such as alteration in cognitive function.

What outputs do you think you will see at the end of this project?

Overall, this proposal aims to find new methods of earlier autism spectrum disorder (ASD) diagnosis and intervention by increasing our understanding of the molecular mechanisms involved in brain formation. This will be achieved by demonstrating that although different factors (e.g. genetic mutations, environmental insults such as maternal infection during pregnancy, pollutions) may contribute to ASD, these factors however, converge onto a similar molecular mechanism, namely alteration of neuronal activity during early postnatal development. We hypothesised that this alteration of neuronal activity has a significant impact on how brains are built, especially on cell types such as the brain immune cell, microglia, which are known to modulate cortical function and behaviour. The discovery from this proposal will be key in (1) identifying the role of neuronal activity in modulating microglia development and number, (2) identifying the critical period during development in which the developing mice are susceptible to changes and (3) convergence of molecular mechanisms leading to the phenotypes typically observed in individuals with ASD. These discoveries will increase our basic understanding as to how brains are built during development and provide insights as to what happens during ASD and possibly other neurodevelopmental disorders.

In the long term, application of the knowledge obtained from this project onto human development will allow for (1) understanding as to how alteration of neuronal activity during early development can impact microglia development and number which in turn may explain some of the impairments observed in ASD, (2) earlier diagnosis of ASD based on changes in neuronal activity observed during gestation or during the first few months of life using resting-state fMRI and (3) new therapeutic avenues that might prevent or reduce the impact of changes of neuronal activity during early development.

To ensure that these discoveries and information will be disseminated as far and wide as possible, we will publish these discoveries in peer-reviewed scientific journals and also in talks and posters during conferences (both positive and negative results). Furthermore, the

project involves the generation of transcriptome datasets, which illustrates the changes in multiple gene expression within the microglia population in reaction to altered neuronal activity. This data set will be made readily available through online repository. We will also summarise our findings for the general public and will communicate our discoveries through press releases and also via social media (e.g. Twitter).

Who or what will benefit from these outputs, and how?

According to the latest statistics from the UK government, neurodevelopmental disorders such as autism spectrum disorder (ASD) affects at least 1% of the population. Unfortunately, this is likely to be an underestimation due to the difficulty in diagnosis, especially among young children. Currently, the earliest detection of ASD in humans are approximately 2 years old. More often however, individuals with ASDs are typically diagnosed much later in life. Earlier detection and diagnosis of ASD will enable earlier intervention that may reduce social and cognitive deficits. One possible avenue that may allow for earlier diagnosis and intervention is by increasing our understanding on the aetiology of ASD.

In the short term, the outputs from this project can be seen in the advancement in basic sciences. The discovery from this proposal will be key in (1) identifying the role of neuronal activity in modulating microglia development and number, (2) identifying the critical period during development in which the developing mice are susceptible to changes and (3) convergence of molecular mechanisms leading to the phenotypes typically observed in individuals with ASD. These discoveries will increase our basic understanding as to how brains are built during development and provide insights as to what happens during ASD. The people benefiting from these outputs will include researchers from within and outside the field where they will be able to utilise the scientific discovery made and the training provided (e.g. knowledge gained from the insights and the direct use of the data that will be made available via publication and online repository). Furthermore, new collaborations may be formed based on the data produced by this proposal.

One of the long-term objectives of the lab is to collaborate with clinicians in order to determine how these findings can be applied onto human development. Application of the knowledge obtained from this proposal will allow for better understanding as to the aetiology of ASD and the possibility of an earlier diagnosis among children. Currently, there are no known treatments available for ASD. Identification of new therapeutic targets that can prevent significant alteration in neuronal activity during development may not necessarily cure ASD, but it will halt the deterioration of further impairments that can occur during development. Consequently, earlier diagnosis and prevention will allow for better management of the symptoms.

Finally, if the outputs from this project can be successfully applied onto human development, individuals with high risk of developing ASD (e.g. siblings with known ASD diagnosis) in the long-term, will benefit from this work as earlier detection of this disorder will allow for more efficacious treatments and preventive measures to be done earlier during development. To this end, we are currently consulting with clinicians in order to determine how the discovery from this proposal can be applied onto human development. Together, this will allow for earlier diagnosis and potentially earlier intervention in individuals that have a high risk of developing ASD.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, we will disseminate the output of the project through publications and preprints. We will also disseminate outputs (successful and unsuccessful approaches) through talks, poster presentations and informal discussions during conferences and meetings. We will also look into building new relationships with clinicians in order to apply this knowledge onto human development. Furthermore, the output of this work can also be further maximised by the formation of new collaborations, both in the field of neurodevelopment and beyond, as the knowledge generated through this project can be easily applied to other diseases, organs and organisms.

Species and numbers of animals expected to be used

- Mice: 5700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 genetically modified mice, both males and females, in this proposal in order to study cell-cell interactions in the developing brain. For this purpose, mice are an ideal choice as brain development in mouse is similar to those in humans the mouse brain contains the same complement of cell types as in humans the availability of genetically modified mice allows for specific cell type manipulation and the induction or deletion of specific gene of interest.

This project will use animals in all life stages. This will allow us to investigate how the brain develops during early postnatal weeks and also to study the consequences of altered brain development in the adult animals.

Typically, what will be done to an animal used in your project?

The bulk of the animals in this protocol will not undergo any further procedures beyond the normal breeding and maintenance procedures (70%).

The remaining animals will undergo at least one (10%), two (15%) or up to three (5%) of the following procedures listed below

Administration of substances peripherally (e.g. via their diet/drinking water, orally, subcutaneously (e.g. under the skin) or intraperitoneally (e.g. into the abdominal cavity)). Substances will be administered maximally for 7 days, twice daily.

Administration of substances directly into the central nervous system of neonates (e.g. intracerebral injections). This procedure will not last more than 30 minutes.

Behavioural tests that aims to measure the different aspects of behaviour that are typically associated with neurodevelopmental disorders (e.g. cognition, compulsion). The maximum length of time the animals will spend performing these behavioural tests will not exceed 24 days.

Brief sensory deprivation/stimulation models aim to elucidate how alteration of neuronal

activity impact on the developmental trajectories of cells present in the developing cortex. These models will be performed maximally for 3 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Previous experience has shown that these procedures do not typically induce any adverse effects. Nonetheless, listed below are possible adverse effects that may arise in this project.

There is a low risk of death occurring from anaesthesia or complications during surgery (<1%)

Other adverse effect from surgeries and substance administration may include weight loss in excess of 15% of total body weight or excessive loss of coat (e.g. piloerection) in rare situations.

Low risk of hypothermia from Morris water maze. This will be mitigated by the use of heating cabinets and by maintaining the water and room temperature appropriately (<1%).

Low risk of pups being rejected by their mothers or foster mothers after administration of substances into the central nervous system (<1%)

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities of the animals in this project will be mild (70%) and moderate (30%).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The main aim of the project is to understand the formation of the brain and elucidate the role of neuronal activity in shaping the development and maturation of the various cell types in the developing cortex.
The developing brain undergoes tremendous changes during the first few weeks of mouse postnatal development. Due to the complexity and ever-changing physiological environment during development, 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. For the same reason, this precludes the majority of in vitro models such as primary slice cultures. Nonetheless, we will use organotypic slice cultures whenever possible to reduce the severity of the procedures. In this instance, we aim to use organotypic slice cultures in order to test the efficacy of our pharmacological candidates before testing these drugs in vivo.

Which non-animal alternatives did you consider for use in this project?

As a non-animal alternative, I have considered the use of organoids as a potential in vitro system. Organoids are self-organising three-dimensional multicellular in vitro tissue. Organoids are derived from stem cells that are meant to recapitulate the developmental progression of an organ in vitro.

In addition, I have checked the FRAME website (www.frame.org.uk) and currently there are no other suitable replacement available for this current project.

Why were they not suitable?

The use of organoids has several major limitations in these key areas as they lack:

the correct cellular structure and layers (e.g. the presence of inputs from brain regions outside of the cerebral cortex such as the thalamus is lacking in organoids)

the complex environment is required to study brain immune cell behaviour and neuronal interaction. It has been previously shown that brain immune cells do not fully mature into the adult state when grown outside the organism (e.g. cell culture and organoids).

The brain consists of many different cell types that originate from different lineages during development. Organoids however are grown from cells originating from a single lineage. Consequently, it is currently not possible to study the interactions between cell types from different lineages using organoids.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have estimated the number of animals used based on my previous experience and the current preliminary data obtained where I have performed power calculations. As most of the data required can be obtained from ex vivo analysis, we will be able to use the same samples for multiple experiments in order to address different scientific questions. The bulk of the animals estimated in this project will come about through the breeding of mice for the appropriate genotypes. The number of animals estimated have been calculated based on Mendelian genetics in order to obtain the desired genotypes. Consequently, multiple breeding will be required to obtain animals with the appropriate genotypes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have 10 years of experience using mice as a model system for studying neurodevelopment. I have used this experience together with tools such as the NC3R's experimental design assistant to minimise the number of animals used when testing our hypothesis, including the use of factorial experimental design. Furthermore, prior to the experimental design, I have also taken the online course (www.3rs- reduction.co.uk) and went through the PREPARE guidelines (https://norecopa.no/prepare) in order 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?

To optimise the number of animals used, all lab members will be required to take an online course designed to minimise the number of animals used (www.3rs-reduction.co.uk).

With regards to animal breeding, we will ensure efficient mouse breeding by maintaining a detailed record of the breeding and colony management. Record keeping in this instance will allow us to identify problems with the colony early on (e.g. the performance of the breeder, rotating breeders on a strict schedule, replacing non-productive breeders). Furthermore, we will also cryopreserve any mice strains that are not in use. We will also have a brain database to ensure that every brain collected will be recorded together with the relevant information (e.g. experiments conducted, age, sex) to ensure that it can be used by the entire lab in addition to other researchers beyond the lab. We anticipate that as the mouse brain is relatively large and quite a substantial number of brain slices can be obtained per animal, this will allow for the maximal use of the tissue collected. In addition, the tissue collected can be used for pilot experiments in which we will test novel reagents such as antibodies. Together, this will reduce the overall usage of mice in our experimental procedures while sharing the resources with everyone in the lab and in the university.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically modified mice to study the function of specific genes in the formation of the brain during development. Some of the procedures that will be used in the project aims to manipulate genes in specific cell types. In the case of neonatal manipulation, we will provide special care to ensure that no pups are deserted after intracerebral injections. In particular, we have previously found that rubbing pups with the bedding reduces the probability of mothers rejecting their pups. This method not only serves as a refinement measure but also lessens the number of animals used, due to a reduction in animal lost. Furthermore, localised administrations and manipulations reduce the adverse effects that are typically associated with systemic manipulations where

multiple organs and cell types are affected at the same time. For all genetic manipulations, animal welfare is our topmost priority and care will be given in terms of anaesthesia, analgesia and recovery monitoring to ensure that any discomfort and suffering will be minimised.

Why can’t you use animals that are less sentient?

The aim of this project is to study brain development which involves a complex choreography of different cell types in an ever-changing environment. Currently, the mouse remains the best options as there are (1) no suitable in vitro experiments that can mimic the in vivo environment, (2) less sentient animals such as drosophila, worms and zebrafish lack a cerebral cortex and in some species (e.g. drosophila) microglia and (3) the brain development process in mice is relatively similar to those in humans.

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. This would include using techniques such as tunnelling. We also aim to minimise any distress in animals while performing behavioural tasks. In the Morris water maze, animals are subjected to 5 trials per day over 7 days where the aim of the test is for the animals to learn the location of the hidden platform. Each trial last for 1 minute followed by a 30 second interval in which the animals are left on the platform. To minimise distress, the water and room temperature will be maintained appropriately and any animals that failed to find the platform after the 1-minute trial will be guided directly to the platform. For rotarod, transient stress may occur when the animal falls from a short distance from the rotating rod. We will minimise stress and avoid potential injury by padding the surface in which the animals will fall onto.

Special care will be given to all animals after a surgical procedure or prior to any animal behaviour experimentation where they will be gently handled and habituated in order to minimise stress inflicted 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. We also aim to minimise the time pups are kept separated from their mothers in order to reduce their distress. All new lab members will be trained on animal cadavers until they are competent to perform the surgeries.

Refinement can also be achieved in the type of mice we used. In full knockout animals, the gene of interest is systemically removed in the entire organism. Sometimes, the global removal of certain genes can induce multiple organ dysfunction that can impact the health and welfare of the animals. By using, specific genetically modified mice in order to manipulate genes in a specific subpopulation of cells when possible, we can mitigate the potential adverse effects that are observed in full knockout animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines and advices provided by the NC3Rs regional manager to ensure that the experiments are conducted in the most refined way. Furthermore, we will follow the guidelines published on the home office websites (https://www.gov.uk/guidance/research-and-testing-using- animals) and the N3CRs

(https://nc3rs.org.uk/3rs-resources).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will be working closely with the NC3Rs regional manager who will be able to advise us on the advances in the 3Rs and implement these advances effectively during this project. Furthermore, prior to the start of any animal experimentations, all lab members are required to undergo a 3Rs training course provided by the NC3Rs (https://nc3rs.org.uk/e-learning-resources) which will provide training on animal welfare assessment, euthanasia, and anaesthesia. In addition, all lab members are expected to be up- to-date on advancement in the 3Rs through literature in journals such as ILAR Journal online and Journal of Applied Animal Welfare Science.

76. Genetics of sex differentiation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

sex determination and differentiation, reproduction, cell fate determination and maintenance, disorders of sexual development, sex hormones

Animal types	Life stages
Chicken	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how cells choose and maintain specific forms and functions (cell states or fates) during development and in the adult animal in specific biological systems, notably the gonads, pituitary and sexually differentiated tissues and how this leads to disease when the processes go wrong.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

During the development of an animal, cells go through a sequence of cell divisions and fate decisions, in which they transition from one cell type to another. These changes are

driven by complex gene expression programs, that respond to their changing environment (external signals). These decisions of cell fate have to be coordinated in time and space to generate functional tissues, organs and the animal. Although some aspects of certain cell fate decisions can be studied i*n vitro*, and we both use and develop such approaches, it is generally essential to study them *in vivo*, using animal models.

The main purpose of the work to be conducted under this Project Licence is to provide fundamental knowledge on gene networks and cell fate decisions in chicken sex determination/differentiation.

Sex determination refers to the process by which a sexually reproducing organism differentiate as a male or as a female. Central to this process is the differentiation of the gonads into either testes or ovaries, which then operate as the niche for the maturation of the germ cells in either sperm or, respectively, eggs, as well as endocrine organs. The hormones produced, the sex steroids, control the fate of other sexually differentiated structures, such as internal and external genitalia and other body features and are also important for fertility.

In mammals sex is dictated by the inheritance of the sex chromosomes (genetic sex determination system, or GSD) to give XX females and XY males. A single gene on the Y chromosome acts as a dominant inducer of testis differentiation. In its absence the ovarian pathway prevails. From its discovery, many other genes have been identified which have allowed to start building the gene networks that regulate the process.

Most of our knowledge derive from the study of mouse models and from the genetic analysis of human patients with disorders of sexual development (DSD): a wide range of conditions present from birth where the development of internal or/and external sex features are, discordant, different from expected, based on the genetic sex (XY or XX); Despite the advances in the field, the molecular cause of the majority of these cases remain unknown.

A critical feature that has emerged and seems to be common to other vertebrates, is that male and female signal networks are mutually antagonistic. One pathway has to be established while the other has to be continuously suppressed.

This makes the system very plastic. For example, in the adult mouse gonad, altering the expression of key male or female promoting genes, such as *Dmrt1* or, respectively *Foxl2*, causes a switch in the gonad sex identity (gonadal sex reversal). Some examples of naturally occurring plasticity have been observed in mammals. For example *Foxl2* has a key role in early embryonic ovary determination in goat, but not in mouse, where it assumes a key role in the ovary only after birth. Seasonal plasticity have also been observed in other mammals. For example in the Spanish mole, the female show sex seasonal variation, as, outside breeding season, part of the ovary produce male hormones, which result in some masculinisation of body features, including a more aggressive behaviour.

Many of the molecular players identified in mammals are conserved in other vertebrates, like birds, turtle, fish, reptiles, however they may be expressed in a different order. This fluidity is quite evident at the top of the sex determination cascade where transitions between different trigger mechanisms have been commonly observed even between closely related species. (e.g. in fish and reptiles it is common to observe transition between GSD and environment-dependent sex determination (ESD)). This means that mechanisms of gonad sex determination are rapidly evolving and therefore are quite

variable across vertebrates, in contrast to most other developmental processes. Even in mammals, that have a stable GSD system, a few species do not have a Y chromosome (e.g. spiny rat), indicating that the regulator may be replaced by other genes downstream in the pathway.

We are far from understanding the sex determination pathways in vertebrates and we need to study different models to understand the core elements of the process.

The chicken has been chosen as, like mammals, it has a stable GSD system (the female inherits the heteromorphic chromosomes (ZZ/ZW system)) and there is a high degree of homology between the chicken and the human genome. It is a versatile model, that is easily accessible for embryo manipulations and now can also be genetically modified. Due to its sensitivity for endocrine disrupting chemicals (EDCs), it has also been proposed as a bioassay for impact assessment of EDCs on reproductive tissues. We aim to understand the molecular mechanism that control chicken gonad sex determination, how the downstream genetic pathways specify male and female different gonadal cell types and how these instructions are coordinate at organ level.

We will use the chick (this PPL) and the mouse (separate PPL) to understand conserved mechanisms and help to construct the networks of gene activity required for ovary versus testis development and reveal how certain genes take critical roles within these network in a species specific manner. By comparing aberrant gonadal fate decision in chicken and mouse models due to variation in genes, developmental programming, or hormones throughout different life stages we will gain understanding on causes of DSDs, on the role of the gonadal sex in controlling the development of sexual dimorphism and the potential consequences of environmental endocrine pollutants on reproduction.

What outputs do you think you will see at the end of this project?

-Improved understanding of the mechanism of chicken sex determination and of the genetic pathways that drive testis or ovary differentiation. We have recently established that the trigger of chicken sex determination depends on the dosage of the Z gene *Dmrt1* and that the gene *Foxl2* is an essential key gene for ovarian determination in the embryo. We now aim to characterize the interactions between *Dmrt1* and *Foxl2* and to identify the networks regulated by these genes in establishing the testis or ovarian pathway. This work may also lead to the identification and characterization of new genes of the network.

Improved understanding of the plasticity of the chicken gonadal sex; by perturbing the expression/regulation of potential key ovary or testis genes in the adult gonad (e.g. *Foxl2*) will lead to new insights into cell fate reprogramming and organogenesis.

Improved understanding of female reproductive function, fertility and premature ovarian failure. We have shown that eliminating one copy of *Foxl2* in chicken results in a phenotype characterized by an eyelid phenotype and, in females, shorter fertility lifespan. These phenotypes resemble the human Blepharophimosis, ptosis, and epicanthus inversus (BPES) syndrome, This model therefore may be used to shed light into human BPES.

-Improved understanding of the hypothalamic-pituitary-gonadal axis. *Foxl2* is a key gene for the adult ovary function, but it is expressed both in the ovary as well as in the pituitary endocrine cells producing the hormones regulating the ovarian cycle. The generation of new chick models where *Foxl2* expression is perturbed in each of these organs separately, will provide new insights into *Foxl2* role in each of these organs and in the sex hormones

local and systemic effects on fertility and may inform new strategies to manage fertility.

Improved understanding of the evolution of sex determination and of the pathways that are conserved.

-Improved understanding of the role of the gonadal hormones and of the sex chromosomes on sexual dimorphism. The chicken model we have generated, carrying a mutation in *Dmrt1*, has shown that ZZ chickens with only one copy of functional *Dmrt1* have gonadal sex reversal (male to female) and sex reversal of the internal and external reproductive organs, but they maintain male body features (e.g. male greater musculature mass, larger combs and wattles, obvious leg spur). This shows that many sexual features of the body in chicken are more influenced by the sex chromosomes of the cells forming the tissues, than by the gonadal hormones. We aim now to analyse the effect of gonadal sex reversal on sexual dimorphism in the opposite direction (female to male, in *Foxl2* knock-out models).

The use of animal models which provide improved understanding of the mechanisms underlying sex bias in human diseases will be of clinical benefit in terms of improved diagnosis and perhaps options for treatments.

We will publish all of our findings in open access journals, with data in a reusable form. Moreover, germ cells from any genetically altered chicken line generated as part of this PPL, will be made freely available to other researchers.

Who or what will benefit from these outputs, and how?

There are likely to be multiple beneficiaries from the outputs above.

-In the short term our studies will shed light into unresolved questions in the field of sex determination and its evolution, opening new ways forward to other scientists working in chicken and other systems. Moreover they will be beneficial also to researchers working on the more applied field of reproductive biology in chicken, but also other birds and mammals

As the chicken is a farm animal, the improved understanding of chicken sex determination will be of relevance to the chicken industry, as it will allow the industry to make more informed decision on issues related to fertility and to sexual development. Similarly our study may be of benefit to conservation programs.
-Our studies on the genetic pathways driving sex determination/differentiation will be of relevance to DSD in humans and have an immediate impact. DSDs represent a major paediatric concern and a significant healthcare burden due to the difficult clinical management of these conditions and, in some, the association with gonadal cancer and infertility. It is estimated that the incidence of new-borns with ambiguous genitalia is 1 in 4,500-5,500 births. More commonly, 1 in 300 new-borns present with some sort of developmental abnormality affecting the somatic sex phenotype. The range of pathologies of DSDs is quite extensive and, as of yet, the molecular cause for the majority of these cases is unknown. Only 50% of the cases receive a definitive clinical diagnosis and only for 20% of those a specific molecular diagnosis. A better knowledge of the genes and genetic pathways involved in the process through the study of animal models is essential to improve the ability of making correct diagnosis of DSDs, and apply the right treatment, which may include surgical intervention and/or lifelong hormonal treatment.

-Improving our knowledge of sex differences may ultimately improve gender based

healthcare in humans, as relevant to the pharmaceutical industry for gender-based drug development In the longer term (perhaps in 5 to 15 years). There are sex differences in food consumption, metabolism and obesity, with concomitant differences in the risk and manifestation of obesity-related conditions such as atherosclerosis and diabetes and in cardiovascular disease and hypertension. It is therefore crucial to determine the factors that lead to the sex differences for the development of novel therapeutics.

How will you look to maximise the outputs of this work?

Alongside the publication of primary research papers in open access journals, we will present our work at meetings, ranging from small focused workshops to large international conferences. Critically, we will communicate negative results and approaches as well as those that are positive.

We collaborate, both internally, within the host Institute, and externally with scientists and clinicians based in the UK and abroad.

We share details of approaches and data generated with our collaborators, which allows for improved development of methods and better synthesis of findings.

Where we develop new transformative methods, we will publish the protocols as papers and post them on bioRxiv.

Species and numbers of animals expected to be used

- Domestic fowl: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use chickens for all the projects covered in this PPL. These studies will be complementary to the studies in mice that are covered by a separate PPL. This is done for multiple reasons. First, these studies will improve our understanding of the evolution of sex determination/differentiation.

There is much to learn about sex determination pathways in vertebrates, including even in mice and humans. Data obtained from the chick will indicate additional conserved mechanisms and help to construct the networks of gene activity required for testis versus ovary development and reveal how certain genes take on critical roles within these networks in a species-specific manner.

Secondly, recent advances in the understanding of sex determination/differentiation have raised doubts as to the universality of the mouse as mammalian model of sex determination. For example, studies in mouse have shown that the gene *Foxl2* has a key role in the adult ovary to maintain the ovarian fate, but it is dispensable in the embryo for ovary differentiation. However in another mammal, the goat, *Foxl2* is necessary for early ovary differentiation. Moreover the differentiation of the early ovary in the mouse is atypical, as it proceeds in the absence of any estrogen and the germ cell niche is only

formed quite late, compared to other mammals. It is therefore important to study other model systems to better understand ovary determination and differentiation. The chicken is chosen because, like mammals, it has a genetic sex determination system and it is an amniote. Most genes important for the differentiation of the ovary or testis are conserved, including *Foxl2*.

The chicken has many experimental advantages. The embryo is easily accessible for manipulations, such as tissue ablations and tissue grafts, administration of substances and gene mis-expression. It is particularly suitable to study gonadal differentiation as the gonad is one of the organs that can be easily and specifically targeted in the embryo. Moreover, it is now possible to generate genome modifications that can be transmitted via the germline.

With respect to life stages, our work ranges from early embryo in the egg, all the way to ageing adults. This range reflects, in part, the focus of the lab on certain genes, for example *Foxl2*, that function in cell fate decisions throughout many or all these stages, but also the importance of understanding the effect of changes that take place during organ development in the adult.

Typically, what will be done to an animal used in your project?

The main work covered by this PPL involves breeding, including genetically altered (GA) animals and harvesting tissues from embryos or from post-hatching animals after they have been killed (using a schedule 1 procedure) for detailed analysis of phenotypes.

Some embryos from wildtype or GA lines may be subject to cell/tissue grafting aimed to generate gonadal chimera. A few of them may be hatched and killed at different stages of gonad differentiation.

Some embryos from wildtype or GA lines maybe administered viral particle, marker molecules, or DNA. Most embryos will be killed before the first two thirds of embryogenesis.

We may use substances such as doxycycline, to induce a genetic alteration, e.g. a conditional loss- or gain-of-function of a specific gene, as well as to follow cell fates, or to isolate specific cell types (e.g. by activation of a fluorescent reporter gene).

We may also use signalling pathway modifiers, such as Fadrozole or Estrogen, to interfere with the sex determination process, or challenge the ovarian or testis pathway and/or use labelling agents, to look at cell processes (e.g. EdU or BrdU for cell proliferation) in tissues after harvesting.

These substances may be administered in the egg, or in post-hatch animals in diet or by injection, and may be carried out multiple times over a few days, followed by a variable period prior to the animal being killed and the tissues analysed.

Most of the manipulation work is carried out and terminated in embryos before the first two thirds of embryogenesis.

Some animals may be used as surrogate hosts to transmit exogenous germ cells that may have been genetically modified. Those surrogates may be GA with defective germ line. We may use drugs to kill the endogenous germ cells. These procedures are done at early embryonic stages and the hatched animals are kept as founders for breeding and for

generating embryos for experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

We are investigating the genetic pathways affecting the development of the reproductive organs, secondary sexual characteristics and the fertility of the birds. Therefore we work with lines with specific gene mutations affecting gene expression or regulation which may result in male-to-female or female- to-male gonadal sex reversal and/or sterility. Those chickens should experience no more than mild effects. The birds will be closely observed to monitor any unexpected adverse phenotypes. With mutations affecting some genes, there may be broader phenotypes, which can lead to more severe adverse effects (e.g. Foxl2 knock-out line has also a chronic eyelid phenotype of moderate severity already present in heterozygotes). All those lines with harmful mutations, or leading to sterility, will be normally maintained for breeding as heterozygotes, or whenever possible, will be maintained by cyclic generation of germline chimeras (chicken surrogate hosts carrying modified germ cells of interest).
Some animals may be crossed to generate homozygotes or compound mutations, where stronger phenotypes occur, including embryonic or postnatal lethality, or reduced lifespan. It will be necessary to study embryonic stages and to keep some animals with harmful mutations until the phenotypes develop, in order to study how they arise. We will kill animals before end points for the relevant severity band are reached.

The frequency, type and severity of any adverse event depends on the procedures being used, together sometimes with genetic status, including the genetic background of the strain. We endeavour to minimise the chances of these occurring; for example we will generate new GA lines by designing genome editing strategies that perturb gene expression in a defined temporal window and/or specific tissue, when the gene under study may have roles in multiple tissues within the body. However the cause of adverse effect sometimes may be unknown. This is also the case of the occasional death after administering signalling modifiers (e.g. tamoxifen).

In the case of new GA lines the phenotype will be analysed in steps, starting with the egg at different stages of embryogenesis and then in the hatched chicken at different timepoints. GA lines with known adverse effects will be closely monitored. One such a line is the Foxl2 knock-out line which has a chronic eyelid phenotype of moderate severity developing from hatching even in heterozygotes and therefore affecting the chick welfare for all its lifespan.

Any animal will be killed prior to it reaching the relevant end points as defined in the protocols.

Surgical procedures are generally performed in the early embryo and therefore should not cause discomfort. All embryonic manipulations may have variable success rate which is difficult to predict. However their outcome is normally established by monitoring the embryos growth, and embryos that continue with their normal development may be incubated up to hatching.

Expected severity categories and the 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 the chicken experiments are:

Mild; about 50% of the animals. Moderate; about 49% of the animals. Severe; less than 1% of the animals.
The germline chimera chickens (chickens derived from embryos injected with exogenous germ cells) and the gonadal chimera chickens (chickens derived from embryos transplanted with tissue that give rise to the gonad) are falling in the mild category. The GA line mutated in the *Foxl2* gene (*Foxl2* knock- out) falls in the moderate category due to an eyelid defect that compromise the ability to blink. Those animals are under eye treatment regime to avoid infections. The severe category includes animals with unexpected severe effects; those animals would be killed if found.

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?

Most, if not all cell fate decisions in the embryo and adult animal take place within a complex environment, where events inside the cells are influenced by a variety of signals, whether these are from neighbouring cells, involve molecules, such as growth factors, cytokines and hormones (which can act over considerable distances), are commensal with the animal, such as gut microbiota, or are part of the environment, i.e. are external to the organism. Moreover, most tissues develop in a complex way in three dimensions over time in a carefully orchestrated manner. Therefore, although some aspects of certain cell fate decisions can be studied *in vitro*, and we both use and develop such approaches, it is generally essential to study them *in viv*o (as a minimum to judge the suitability of *in vitro* systems to give meaningful information).

Several distinct cell lineages give rise to the developing gonads and their continued interactions are required for appropriate gene activity leading to the development of either testes or ovaries. It is currently not possible to mimic all of these cell-cell interactions using cells maintained *in vitro*. Moreover even if we can culture the intact early gonad for periods of few days, which does allow us to follow some events in real time (and reduce animal numbers), this still requires breeding to produce the animals.

To study postnatal gonadal sex reversal, cannot meaningfully be studied in any current *in vitro* system. While it is possible to culture isolated specific type of ovarian cells for a limited time, they tend to lose expression of critical genes and their normal features. The same is true of cells in the testis.
Without a robust and reliable culture system that could maintain the properties of different cellular types, it would be impossible to address the consequences of deleting *Foxl2* in the adult ovary in meaningful way. Moreover, it would not be possible to investigate how other

testicular cell types differentiate, nor the complex morphological changes occurring from ovary to testicular-like structures.

(iii)The pituitary develops through a complex series of events involving different tissues and there are no actual protocols to reproduce its cellular organisation and proper hormonal function *in vitro*.
Progresses have been made in growing pituitary organoids, from stem cells, however also these structures don't have sufficient complexity to model the *in vivo* organ, or to be useful to address the complex interactions of the pituitary with the gonad, to regulate reproduction, ageing and other key physiological processes, which requires whole animal study.

(iv). There is now increasing evidence that many aspects of anatomy, physiology, behaviour, pathologies and responses to treatment, differ between the sexes and even when these appear similar, the underlying mechanisms may be different. These differences are likely to be due to direct effects of sex chromosome-linked genes, to sex hormones made by ovaries or testes, or both. Moreover, these effects can be organisational (i.e. they are established during development, perhaps prior to any obvious difference), or activational (require constant input). Experiments to understand the mechanisms involved, the importance of which have been widely recognised in recent years, cannot be conducted *in vitro*.

The use of the chicken as a model species itself addresses "replacement". The chicken offers advantages over using a mouse model, as the mother does not need to be killed to obtain the embryos and, as germ cells can be introduced in the embryo, there is no need of any surgical manipulation of the hen to generate new GA lines. Moreover the majority of experiments to study sex determination will also be carried out at unregulated developmental stages (before day 14.5 of incubation).

Which non-animal alternatives did you consider for use in this project?

We complement our *in vivo* analyses in chicken with tissue culture models and organ culture.

Why were they not suitable?

We can gain a certain amount of information from these culture systems, but there are serious limitations. It is not currently possible to replicate the complexity of tissue structures in culture models. Moreover such cell and organ cultures cannot permit research on aspects of biology such as reproduction or 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?

This estimate is based on several factors. It is based on our experience of working with chick embryos and the experience over the past 4 years with generating and maintaining chicken lines (e.g. *Foxl2* and D*mrt1* knock-out GA lines).

We also continually re-evaluate the numbers of animals required for each experiment. This will allow us to determine the number of animals required per experiment to give statistically valid results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing specific experiments within the overall project, we estimate the minimum number of animals required to give robust answers. Most often this can be based on our prior experience, or on published data. Often it is not necessary to use statistics (e.g. three transgenic lines giving the same result shows that this is correct), however, we perform statistical analysis whenever necessary. Where experiments result in consequences, for which we have little or no prior information, usually around 5 or 6 animals per treatment group (which will include sex as a variable when relevant and possible) are sufficient to obtain robust results. The design of quantitative experiments generally follows the ARRIVE guidelines and sample sizes may be set using power analysis. Any exceptions are where there is a degree of variability beyond our control (for example, where minor fluctuations in conditions together with threshold effects require more animals to be examined in order to have statistically significant results). We generally use a significance level of 5% and a power of 80%, estimating standard deviation from pilot experiments. We may include advice taken from local statisticians as well as make use of online tools, such as the NC3Rs' Experimental Design Assistant. For some important questions that we wish to address there can be a choice between using a mild procedure but many animals because the measurable effect is weak, or a moderately severe procedure with few animals because the effect is robust. Our choice will depend on the specific question and available resources, but it will most often be to use fewer animals.

Genetically altered chicken embryos depleted of their own germ cells will be used whenever possible as surrogate hosts of exogenous avian germ cells to produce chicken chimera founders. The transmission rate in these GA hosts is 100% allowing to reduce both the number of chimera founders and the number of offspring for the establishment of the line. This strategy is more efficient than using host surrogate embryos treated with chemotherapeutic reagent to reduce the number of the host germ cells and improved transmission of injected germ cells. Moreover, these germ cell chimeras can be used not only as line founders, but also as regular providers of eggs for experimental studies and may be the method of choice to maintain a line with a harmful phenotype or a phenotype that leads to sterility.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We try to keep as few chickens as possible by carefully monitoring our flocks and by good practice.

Whenever possible and when there are no harmful phenotypes (or infertility) we maintain GA lines as homozygotes to reduce the numbers of animals required for experiments and to reduce the need to genotype.

In case of infertility or harmful phenotype we may maintain the line via the germline chimera founders.

We may also make use of fluorescence reporters that can (in some circumstances) avoid the need of genotyping.

The ability to generate transgenic lines using primordial germ cells which can be cultured and frozen down means that lines of chickens would no longer need to be held and bred indefinitely. Frozen germ cells can be used many years later when a particular breed of chickens would be again needed by researchers.

Whenever possible, we pre-screen substances (including molecules to induce gene expression, cell death, mutagens, etc.) and agents such as viruses, in the egg (*in ovo*), or in cell culture (*in vitro*) to determine approximate doses required. We also test genome editing components *in vitro* (e.g. in primordial germ cells), prior to the generation of genetically altered animals.

To maximise information gained from single animals, we use live imaging when feasible (e.g for embryo work), obtain data on as many tests of behaviour as possible on single animals, and obtain relevant tissue samples from multiple sites after killing, where more than one project involves the study of an animal with a particular genotype; for example as *Foxl2* is relevant to studies of the gonad, pituitary and eyelid, we often collect multiple tissues from single animals.

Similarly, when designing new genetic tools, and maintaining animals derived with these, we will, wherever possible, do so in a way to allow them to be shared amongst as many people as possible, including making use of the Institute sharing platform. This efficient use of animals minimizes the number used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

-Cultured germ cells that can be genetically modified to disrupt gene function and used to produce chimera founders is unique to the chicken. The germ cell transplantation to form chimeras are carried out at early embryonic developmental stages (unregulated stages) and in an animal model that does not entail a surgical operation on the mother, or her death, to provide access to the embryos.

Moreover our use of genetically modified surrogate hosts for the generation of germline chimeras results in 100% transmission of injected germ cells, reducing the number of chicklings to be screened and making these chimeras also good providers of fertilised eggs for experiments. The latest refinement of the protocol, which allows to generate chimera founders (G0 generation) from both sexes of GA surrogates, may eliminate the necessity of

breeding the siblings (G1 generation) for line maintenance.
This is particularly important if the genetic modification causes infertility or if it adversely affects multiple tissues.

-Sex determination is an embryonic process so many experiments can be carried out in the egg.

-We choose well-established protocols, known to have minimal harmful effects, whenever possible. Many of the genetic and physiological manipulations, as well as the administration of substances, including gene inducers and repressors, viruses, cells and grafting of tissues, are standard and previous refinements from the literature will be used, if possible. For novel types of manipulation, or where insufficient information is available, small-scale pilot experiments are conducted in order to determine the best conditions to obtain a sufficiently robust and meaningful response from the minimum dose, exposure time, or treatment. These pilot experiments help to minimize any potential suffering.

-Although it is not always possible to predict the nature or severity of any defect that arises from a newly generated genetic alteration, we take steps to minimise unwanted phenotypes and/or the number of animals exhibiting these. For example, we may make use of tissue-specific regulatory elements and whenever practical, we may make genetic alterations that are inducible, so that the animals do not show a phenotype until expression of the candidate gene or a deletion is induced.

-When the experiment is predicted to lead to harmful effects outside the body system under study, we will provide treatments designed to alleviate these; for example the eyelid phenotype present in chickens mutated at the *Foxl2* locus is constantly monitored and regular ointment treatment is provided.

-To minimise stress during breeding and maintenance we follow best practice guidelines, institute refinements and, for some strains, our own specific procedures of husbandry. (e.g. the *Foxl2* knock-out line is in pens layered with horse shavings to minimise dust). In the case of any new strain of animal or application of any new procedure or refinement we pay special attention by increased observation and monitoring starting with the embryo and then in the hatched chicken, until we have become familiar with the phenotype and/or the consequences. If welfare implications are identified they will be acted upon and refinements considered in consultation with the NVS, NACWO and animal technicians.

Why can’t you use animals that are less sentient?

We use the chicken as a model complementary to the mouse, to study sex determination/differentiation, partly for evolutionary comparisons, partly because some aspects of the process may be more similar between chicken and human than mouse and human (e.g some features of fetal ovary differentiation) and partly because certain embryological techniques are feasible *in ovo*, but not in utero. A significant fraction of our research involves studies on chicken embryos *in ovo* prior to two-thirds through embryogenesis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all manipulations, we will adhere to the relevant guidelines that aim to minimise suffering. We examine the animals for signs of pain and discomfort (such as prolonged

lethargy) and monitor body condition, killing the animals if the distress is likely to be more than temporary. Many of the genetic and physiological manipulations, as well as the administration of substances, including gene inducers and repressors, viruses, cells and grafting of tissues, are standard and previous refinements from the literature will be used and added to ifpossible.

For novel types of manipulation, or where insufficient information is available, small-scale pilot experiments are conducted in order to determine the best conditions to obtain a sufficiently robust and meaningful response from the minimum dose, exposure time or treatment. These pilot experiments help to minimize any potential suffering.

Appropriate aseptic surgical techniques, temperature, and fluid therapy, will be applied as necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

These will include publications from the NC3Rs, the Institute for Animal Technology and the BVAAWF/FRAME/RSPCA/UFAW Joint working group, but also relevant articles in scientific journals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay up to date via regular communication with animal care staff at the Institute, other scientists in our fields, via e-mail and other updates and publications from, and occasional attendance at meetings held by, the NC3Rs, the Institute for Animal Technology, and the International Society for Transgenic Technology, and through regular visits to their websites: https://www.nc3rs.org.uk/3rs-resources https://www.transtechsociety.org/ https://www.iat.org.uk

77. Modelling Liver Disease 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

Fibrosis, Liver Cirrhosis, Therapy, Chronic liver disease, Liver damage

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to test the effect of novel compounds in models of liver disease, in order to assist with the development of new therapies where none exist or current treatments are inadequate.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Liver disease is now the third leading cause of premature death in the UK and is the top cause of premature death in individuals aged 35-49 years old. In contrast to heart disease

and cancer, where death rates have remained relatively stable, deaths from liver disease are increasing. If detected early, the effects of liver damage can be reversed successfully with treatment that can allow the liver to regenerate. Unfortunately, the majority of individuals already present with advanced liver scarring, at which point treatment options are limited.

Fibrosis (or scarring) is a result of part of the liver's healing response to damage and often occurs in the absence of any obvious symptoms. Long-term liver damage can lead to progressive fibrosis and eventually cirrhosis, liver failure and premature death. There is an urgent need for new therapies to prevent, slow or reverse fibrosis (and therefore cirrhosis) in patients with chronic liver damage and to ultimately prevent liver failure and death. Despite this need, there are currently no licensed treatments to slow or prevent fibrosis in chronic liver disease. Furthermore, liver transplantation is often the only treatment option for advanced cirrhosis, and with a shortfall in donors for liver transplantation, there is an urgent need for therapies that are able to prevent progression to this stage of disease.

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 at our establishment 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: 6350
- Rats: 825

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 shall predominantly use mice, but rats could also be used if the drugs being tested are known to be more effective in rats than mice. 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 the majority of the genetically-modified animals used under this licence will be humanely culled to provide cells and tissues for use in the laboratory and will undergo no further experimentation. Where experimentation is conducted, procedures include manipulation of diet or administration of substances to induce liver injury where clinical signs should be minimal. All animals will be humanely killed at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

To replicate the effects of human chronic liver disease, listed models may lead to clinical signs including jaundice, ascites (abdominal fluid), or weight loss. Animals will be closely monitored throughout for signs of distress and culled humanely if they exceed the predefined limits.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of the animals used will experience mild to moderate effects. Any observed effects should be short in duration, and direct effects from liver injury should be mild. The majority of these effects are associated with weight loss, fatigue or mild discomfort following administration of treatment.

What will happen to animals at the end of this project?

- Culled

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 dampen the complex biological processes seen in liver disease.

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 liver fibrosis 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 Contract Research Organisation, 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.

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.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 liver disease. *In vivo* models are combined with advanced *in vitro* assays that allow us to study the effects of the test drugs on the processes involved in the onset and progression of liver fibrosis that often lead to liver failure.

The majority of the genetically-modified mice will be humanely culled to provide cells and tissues for use in the laboratory and will undergo no further experimentation. Beyond our breeding program, the majority of the animals used will experience mild to moderate effects. Due to the often asymptomatic nature of liver disease, the majority of the models of liver fibrosis and injury we will use are also associated with mild effects. Where possible, models that are able to provide the most relevant answers in a short time-frame will be used in order to minimise the duration of any clinical signs.

Why can’t you use animals that are less sentient?

Rodent models are extremely well-characterised and validated for studying the progression and regression of liver fibrosis 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 liver disease.

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 destroyed. 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).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to following the ARRIVE 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 our Establishment 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.

78. Breeding and Maintenance of Immunocompromised and Genetically Altered Mice as a Service

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, breeding, colony management

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 licence is to breed, maintain and supply high quality immuno-compromised and genetically altered mice for use in cancer research under the authority of other project licences at our establishment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our breeding programmes can be managed in dedicated facilities and closely monitored to minimise over-production and to produce a high quality healthy animal. We work within guidelines which are taken from the Genetically Altered breeding initiative from the Home Office in relation to colonies which will be held under this service licence. Our experienced animal technologists supply the specific expertise necessary to provide the optimum

husbandry conditions, the highest level of health and welfare as well as accurately maintaining the minimum colony size to produce the animals required for research.

As a direct result of the work that is carried out in our facility using these strains of mice, major and innovative developments in the treatment of breast, testicular, lung and gut cancers have been made in human patients. These have been widely publicised in the media in recent years.

What outputs do you think you will see at the end of this project?

A single centralised breeding project will be managed by someone with specialised technical expertise in the required breeding methods and with stringent colony management. This is administratively efficient and has welfare benefits such as reduction in animal numbers by effective liaison with end- user projects. This will also ensure that 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.

There has been a proven record of efficient generation and breeding of Genetically Altered Mice and this Licence will continue and improve the provision of high quality animals to researchers whenever such strains are not readily available from commercial breeding establishments.

Who or what will benefit from these outputs, and how?

As a direct result of the work that is carried out in our facility using these strains of mice, major and innovative developments in the treatment of breast, testicular, lung and gut cancers have been made in human patients. These have been widely publicised in the media is recent years.

How will you look to maximise the outputs of this work?

We will utilise published best practice guidelines for the breeding and management of genetically altered mice.

In addition we will liaise closely with research teams to plan their colony production to provide the numbers of animals they require whilst reducing the over production of animals.

Species and numbers of animals expected to be used

- Mice: 27550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 that lack a fully active immune system are a valuable research tool in cancer research enabling human tumour material to be grown. Genetically Altered mice are also a

valuable research tool enabling mice with specific targeted genes to be bred and maintained for use in many areas of cancer research. Many of the research projects will involve the use of laboratory systems such as cell culture, human tissue assays and computer modelling to complement the animal work. However, Laboratory assays cannot adequately replicate many of the complex molecular, cellular, physiological and behavioural properties necessary to fully understand how genetic modifications result in normal or abnormal tissue growth.

For this project adult mice will be bred in efficient colonies. The offspring of these breedings will be transferred to other Project Licences at the Establishment for use in cancer studies.

Typically, what will be done to an animal used in your project?

For this project, adult mice will be bred in efficient breeding colonies. The offspring of these breedings will be transferred to other Project Licences at the Establishment for use in cancer studies.

Typically, the only procedure these animals will be exposed to is identification by ear marking.

What are the expected impacts and/or adverse effects for the animals during your project?

Some of the genetic alterations will cause tumour growth in some animals and these will be closely monitored for typical signs of pain or distress. Mating of mice does not typically have any adverse effects. Occasionally, young pups are injured or killed by their parents. This we try to reduce by providing an enriching environment and ensuring that weanlings are removed before new pups are born. Occasionally we remove Male animals to reduce the likelihood of injury

Expected severity categories and the 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 animals in these protocols is Mild

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 use of animals in cancer research remains a vital tool in improving our understanding of how biological systems work in disease, and in the discovery and development of new

medicines, treatments and technologies. This licence aims to breed and maintain animals with impaired immune systems and genetically altered animals for use in research projects at the establishment.

Detailed justification for the particular programmes of research is provided by the individual scientists in their own licences that must always be approved by the Home Office.

Which non-animal alternatives did you consider for use in this project?

Many of the research projects will involve the use of laboratory systems such as cell culture, human tissue assays and computer modelling to complement the animal work.

Why were they not suitable?

Laboratory systems cannot always adequately replicate the complex molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal tissue growth.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Research into the causes and subsequent treatment of a wide range of cancer types requires the use of specific strains of mice including those with impaired immune systems and animals with specific genetic modifications that are relevant to the genetic pathways now known to be associated with tumour development.

We estimate that we will breed and supply 5000 immune deficient mice each year, approximately 500 Genetically Altered mice per year and very small numbers of approximately 10 animals for Superovulation per year.

These figures are based on projected animal supply to other project licences at the Institute. Supply and demand vary considerably however and historical data is also used to estimate future trends in usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Using the latest good practice guidelines on the breeding and management of laboratory mice will allow us to maintain the lowest number of breeding animals to produce the required number of study animals.

Unfortunately due to the complex genetics involved in the breeding of Genetically Altered mice, there will be animals produced that we are unable to use for our studies. These we will humanely killed at the earliest opportunity or used for collection of tissue and blood products for further use in laboratory research.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Unnecessary production or import of genetically altered animals will be avoided by searching cryobanks and databases. Examples of resources available include:

NC3R's breeding and colony management: https://nc3rs.org.uk/breeding-and-colony-management

Animal Welfare Management Discussion Group (AWMDG): An email network for Named Persons to share ideas

RSPCA Animals in Science Resources:
https://science.rspca.org.uk/sciencegroup/researchanimals PubMed:
http://www.ncbi.nlm.nih.gov/
Web of Knowledge: https://wok.mimas.ac.uk/

Jackson laboratory: http://www.jax.org/ & http://jaxmice.jax.org/index.html

Home Office Technical Advice: https://www.gov.uk/guidance/animal-research-technical-advice

The strain used for generating a new colony will be carefully considered to avoid producing unwanted mice. Animals will only be bred if a user requirement has been established, and the breeding programme will be subject to regular review to optimally meet anticipated demand. Spare animals will be made available for use on other scientific projects.

Breeding will be optimised, wherever possible, to produce only the genotype required e.g. Homozygous breeding pairs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 universally 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.
Careful analysis of the genetics of the animals allows us to only breed those animals that will not experience any adverse effects during their breeding life. For example we would not breed female animals that are predisposed to develop spontaneous tumours.

We also maintain all animals under strict environmental conditions to prevent infections

from unwanted pathogens. The methods chosen are all standard for this type of work.

Why can't you use animals that are less sentient?

Much of the work carried out at the Establishment is designed to mimic disease in human patients. Indeed much of our work involves the use of human tissue to see how novel treatments can be used to provide a clinically relevant treatment regime for patients with that type of disease. This can only be replicated in live mice as the tumours will only grow when complete body systems are supporting them.

There is also a great deal of laboratory analysis that can also be carried out that would not require the use of a live animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Routine health screening of the existing colony and careful examination of the health status of any new animals acquired from external collaborators ensures that the health quality of the colony is maintained at a high level.

Where there is a need to identify the mice , refined methods of tissue sampling will be employed ie: ear- marking and surplus tissue used for genotyping.

Some of our strains of mice have impaired immune systems due to their genetic modification. This allows the growth of human and other non-mouse tissue as the animal's natural immunity is unable to reject the foreign material. These animals demand special care and our husbandry and care routines ensure that these animals are maintained in a healthy environment

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Published guidelines for best practice will be followed, including:

Refinement and reduction in the production of genetically modified mice; Laboratory Animals Vol 37, Supp 1 July 2003.
Breeding and Colony Management (NC3Rs) https://nc3rs.org.uk/breeding-and-colony-management Assessing the welfare of genetically altered mice. Wells et al (2006) Laboratory Animals 40(2), 111-114 Home Office Technical Guidance: https://www.gov.uk/guidance/animal-research-technical-advice
How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Wherever we can we replace the use of animals in research, refine experimental procedures and minimise the number of animals used in experiments. We work closely with organisations such as the National Centre for 3Rs (NC3Rs) to work on new approaches and technologies to minimise the use of animals and improve animal welfare.

79. Xenopus as a model for drug development and toxicology

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

Xenopus, Drug Development, Toxicology, Cardiotoxicity

Animal types	Life stages
Xenopus laevis	adult
Xenopus tropicalis	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?

As part of the drug discovery pipeline a new drug's potential toxicity is tested at the whole animal level in mice. This uses a lot of mice and is costly. In the spirit of the NC3Rs we are developing protocols that would use frog embryos to test toxicity and so flag up harmful/toxic compounds at an earlier stage in the drug development 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?

Toxicology research may lead, in the long term, to fewer experiments on higher vertebrates such as mice and rats as the methods developed will identify and flag potentially toxic drugs earlier in the drug development pipeline.

This project will therefore be important in many areas of biomedical research including stem cell biology, cancer biology and toxicology

What outputs do you think you will see at the end of this project?

We hope to develop new assays to look at compound toxicity. These assays will be presented to Pharmaceutical companies either in the form of publications or personal talks with the idea that they may adopt the assays as part of their drug discovery programs.

Who or what will benefit from these outputs, and how?

In the short term the beneficiaries of our work will be students and other researchers trained in the lab to be the scientists of the future.

Our work will also be of interest to researchers working in the fields of Drug evelopment, Cell Biology, Cancer Biology and Toxicology. In addition some of our projects have direct implications on the NC3Rs.

How will you look to maximise the outputs of this work?

I will endeavour to publish the groups work wherever possible in appropriate journals. Myself and others in my lab will attend relevant conferences and present our work in the form of invited talks or posters.

I am in touch with some colleagues working in the Pharmaceutical Industry. I will reach out to Pharma with respect to our drug discovery and development objectives.

Species and numbers of animals expected to be used

- Xenopus laevis: 25-50 adults.
- Xenopus tropicalis: 20-50 adults

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using the tadpoles of Xenopus laevis (the African clawed frog) and Xenopus tropicalis as model organisms. We use Xenopus because the females can be induced by a simple injection to lay eggs all year around when kept in an ideal environment and this provides us with a source of embryos (frog spawn). During the course of a year a colony of approximately 150-200 frogs will be induced to lay eggs 1-3 times. The better the quality of care of the frogs the better the quality of the eggs/embryos they produce. To therefore minimize having to repeat experiments it is essential to have healthy animals.

The Xenopus embryos develop outside the mother and they are large which makes observation and manipulation relatively easy. Tadpoles have hearts, livers, brains, eyes and kidneys just like humans. In fact a tadpole with a fully functioning heart, which can be observed beating using non-intrusive methods, develops from a fertilised egg within 4

days. This makes them ideal for studying the effects of compounds on organ function. Embryos produced will be used to study questions of toxicity using a variety of methods. Therefore, what we learn in tadpoles about drug/compound induced changes to basic cellular and molecular developmental mechanisms will enhance our understanding and identification of toxicity in humans.

Typically, what will be done to an animal used in your project?

Female frogs from whom we need to obtain eggs are injected with PMSG 4-7 days before the eggs are needed in order to prime the animal to generate good and healthy eggs. The day before an experiment the females are further injected with HCG (Human Gonadotrophic Hormone) which induces them to lay their eggs 12-18 hours later. Each female is very gently massaged to get her to lay her eggs in a petri- dish. This procedure is carried our 1-6 times over the course of a day with a minimum of 1 hour rest between squeezes. The female is then observed for a further 12-18 hours to make sure they are fine before being returned to the frog facility.

Males (laevis and more commonly tropicalis) are injected with HCG to make them more fertile before they are sacrificed and the testes removed in order to carry out in vitro fertilisation of the eggs.

Note - Commercialy bought Frozen sperm are increasingly being used which means that over time the number of Males being used will decrease.

Tadpoles that are treated with various compounds will be killed humanely after less than 10 days.

What are the expected impacts and/or adverse effects for the animals during your project?

The procedures used are mild and next to no adverse effects are expected with the procedures used.

For affected tadpoles there will be diverse adverse effects including potentially: heart or circulation defects, craniofacial defects, altered behaviour, gut defects limiting the ability to feed properly and there may even be alterations in overall body structure. These tadpoles are normally killed humanely after less than 10 days.

Expected severity categories and the 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 procedures on the female Xenopus and male Xenopus during induction of laying of eggs and fertilisation of the eggs is mild. When used for natural mating the females and males can be reused after a suitable recovery time (minimum 3 months) They can be used in this way multiple times until they reach an age when they no longer produce eggs of a suitable quality (approx 10-15 years of age). At this time they are euthanized. The proportion showing an adverse effect is approx. 0.5-1%.

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?

With respect to our chemical screens and toxicity work the use of the whole organism helps with identifying compounds that have toxic or other effects which might not be the case with screens involving simple biochemical, cell based and organoid assays.

Which non-animal alternatives did you consider for use in this project?

Tissue culture, organoid culture, computer simulations

Why were they not suitable?

Responses and changes in response to toxic compounds can occur due to interactions between different cells and tissues. Cell based assays generally use only one cell type so are not a good model in this respect. Organoid cultures can sometimes include more than one cell type but are still limited and do not include a functioning vasculature.

Computer simulations are increasingly being used in toxicology but in all cases in vivo experiments do at some point need to be done to validate and test predictions and 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?

These are based on the numbers needed from past experience and from preliminary experiments, which have effectively been pilot studies for this investigation. Importantly many of the embryos that will be used in this project can be deemed as spare embryos leftover from experiments being carried out in the lab on other projects. In which case the numbers of animals used will be significantly lower than would otherwise have been.

Adult Animals: When the lab is running at maximum efficiently we plan to do 1-2 Frog experiments a week. One frog experiment involves priming, inducing and collecting the embryos from 3-6 frogs. We usually collect eggs most weeks (approx 40 weeks a year) for our research on early development.

Spare embryos not used in these experiments will be grown to stage 38 where they will then be used for this project. Frogs can be used after a minimum of 3 months. Therefore over a year we would use up to 12 frogs per week for 40 weeks = 480. Frogs can generally be reused 3 times in a year so 480 divided by 3 = 160. So at maximum efficiently we would look to use 160 female frogs in a year.

We do fewer Xenopus tropicalis experiments (approx 20 per year) so need fewer frogs and tadpoles

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each week we have a lab meeting of all the Xenopus users at which we determine what people are planning for the following 1-2 weeks. This way we can plan experiments where personnel share the eggs/embryos produced and so maximise the efficiency of one experiment. Occasionally experiments will be delayed or bought forward in order that one day of egg collection can be carried out instead of two.

At this current time we are transitioning to using commercially bought frozen sperm instead of live males for in vitro fertilisation of eggs. This will not fully replace using Males but will drastically reduce the number of Males we do use.

With respect to the numbers of tadpoles used to develop screening protocols we will be driven by what is required statistically. We will discuss with statisticians and determine the minimum number of tadploes required for each procedure to make the result significant. This is ongoing work. As the assay is developed we will then be able to fine tune it for maximum efficiency and thus reduce the number of tadpoles used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our Xenopus facility is designed to keep the frogs as healthy as possible. The healthier the frog the more likely she will produce good quality eggs. If we can be sure of obtaining good eggs we can reduce the number of females we inject for each experiment. We are continually looking for ways to improve the care of the frogs in a cost effective manner. We are planning over time to initiate the chipping of the frog colony. This will enable detailed records to be kept for each frog. those that routinely provide bad quality eggs will be culled to improve the efficiency of the system.

Importantly many of the embryos that will be used in this project will be spare embryos leftover from other experiments being carried out in the lab as part of other projects. In which case the numbers of adult animals used will be significantly lower than would otherwise be predicted.

The Xenopus community in the UK and the rest of the world routinely discusses issues of care and welfare for the animals at meetings such as the British Xenopus group meeting and once a year for the International Xenopus meeting or Xenopus PI meeting in the USA. Web sites such as http://www.xlaevis.com/ also provide updated information. I am in routine contact with colleagues elsewhere in the UK and the rest of the world who provide help and assistance on best practice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Xenopus laevis, Xenopus tropicalis We will use Xenopus because:
Large numbers of Xenopus embryos are produced externally and obtaining them requires only a mild procedure (injection of HCG into the female).

We will use tadpoles prior to the protected age, when the brain is poorly developed and lacks the region that, at least in man, generates the distress associated with pain.

The tadpoles are largely transparent, allowing simple observation of many of the phenotypes produced without invasive methods.

Why can’t you use animals that are less sentient?

We are studying the effects of compounds on animal physiology and require embryos to do this. Xenopus laevis and Xenopus tropicalis embryos/tadpoles develop outside the mother which is beneficial with respect to observing phenotypes compared to mice.

The frog's genome is very similar in structure and detail to the human one and more than 80% of the currently-identified human genetic disease genes are found in it. To use a less sentient animal in which the genome is sufficiently well annotated for these studies means using zebrafish or Drosophila. The latter, in many cases, lacks sufficient protein identity to humans to make it possible to re-create human disease phenotypes. The structure of the zebrafish genome is very different from that of humans/frogs.

In terms of studying toxicity. Zebrafish only have a two chambered heart compared to the 3 chambered heart of Xenopus. the Xenopus heart is therefreo closer in morphology and function to mammals compared to zebrafish and therefore a likely better system for identifyig toxicity that would also be found in mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We mainly propose to use Xenopus laevis and Xenopus tropicalis for our experiments as they are a well characterised and well used model organism for vertebrate development. They are relatively easy to keep and the adults are subjected to only mild procedures.

The compounds that will be tested on the tadpoles will have already come through preliminary screens for toxicity such as tests on cells lines and organoids grown in culture and computer based predictions. Any tadpole(s) which show undue stress when exposed to a compound will be humanely euthanised. The experimentwill then be evaluated and reviewed accordingly

As mentioned before we are gradually transitioning to using frozen laevis and tropicalis sperm for in vitro fertilisation. This will in time lead to needing less males in our facility.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Online protocols are published by many Xenopus labs and are often available on lab websites. Xenbase and the Xenopus Stock Centres in the UK and the United States also publish standard protocols. Online forums and the biannual International Xenopus Meeting and Xenopus PI Meeting also allow for free exchange of knowledge and best practice.

In addition Cold Spring Harbor Protocols publishes may papers in the 'Xenopus Collection' (http://cshprotocols.cshlp.org/cgi/collection/xenopus) which provide an up to date resource.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am in regular contact with colleagues in the UK and the rest of the world from whom the latest news and advances in Xenopus research can be learnt. The Xenopus community has an online news magazine called Xine from which news is obtained. In addition the Xenopus community in the UK and the rest of the world routinely discusses issues of care and welfare for the animals at meetings such as the British Xenopus group and once a year for the International Xenopus meeting or Xenopus PI meeting in the USA. Web sites such as Xenbase (http://www.xenbase.org/entry/) and http://www.xlaevis.com/ also provide updated information.

80. Mechanisms influencing mammalian digit tip regeneration.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Regeneration, Digit Tip, Blastema, Stem Cells, Mammalian

Animal types	Life stages
Mice	neonate, adult, embryo, 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 is to identify the signals in the environment that are important for successful regrowth of the tips of fingers and toes (called digits) that have been amputated in mammals and to understand how cells in the digit respond to those signals. I aim to learn if we can use this information to promote regeneration in tissues where it doesn’t normally occur.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 over 25,000 people in England currently living with limb amputations mainly as a result of illnesses such as diabetes or traumatic injury. If you cut off a person’s arm, it won’t grow back. Instead, the wound will heal and you will be left with a scar. Surprisingly, however, removing the end portion of a person’s fingertip does not scar. It will completely regrow all of the missing tissue. Understanding why these two different responses occur in a human could help provide insight into how we can regrow other parts of the body.

What outputs do you think you will see at the end of this project?

The main aim of this project is to study how cells in the mouse digit tip respond to injury. We are trying to understand how these cells are able to regrow the digit tip perfectly in order to uncover the basic principles that guide successful regeneration. The results of this study will help us understand how some tissues heal without scarring and will help other scientists working in the regenerative medicine field.

This project will likely result in new information and publications about the following:

Understanding how cells in the mouse digit tip communicate with each other and the cells that surround them (tissue regeneration). The mouse digit tip, which regrows upon amputation, is a powerful system for investigating how tissue repair and regeneration works. This process is similar to human fingertip regeneration and functions as an excellent substitute. In both mice and people, digit tip regrowth depends on how much of the finger or toe is removed. If you remove more than 60% of the end of the digit, it will fail to regrow. This is because a key structure made up of a clump of cells capable of growth fails to form. This key structure is called a blastema and it contains all the cells that will regrow the missing parts of the finger or toe. This project will aim to understand which growth factors or molecules in the injured digit tip environment are important for telling cells to form the blastema, and where these signals come from.

Understanding how the DNA is modified (epigenetic changes) during mouse digit tip regeneration (development & tissue regeneration). Most vertebrate embryos have the ability to regenerate their limbs. After birth, as animals and humans mature, this ability is gradually lost. There is, however, one exception to this rule. In both mature rodents and humans, regeneration occurs when the end of the digit tip is removed. One reason for this remaining regenerative capacity may be due to changes to the DNA, called epigenetic modifications, that allow genes to be turned "on" or "off". This project will aim to understand the epigenetic modifications that occur in response to injury and how these differ from early embryonic development.

Who or what will benefit from these outputs, and how?

One challenge for scientists is to take the knowledge that they discover and make it into therapies that benefit people. The first step in this process is to identify which factors promote regeneration in mammals. Then we need to determine if we can add these factors to the injury or help the person to produce them naturally so that their body will regenerate. This study will aim to answer 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 organizations such as Diabetes UK. This will mostly benefit the scientific community. *In the long term,* this information could be used to develop therapies to improve repair and regeneration in human tissues. This research is expected to provide targets for use in regenerative medicine. These targets may be important for people who suffer with diabetes mellitus (Type 1 diabetes) who need to have their fingers/toes and/or limbs removed because their illness was not managed well.

How will you look to maximise the outputs of this work?

To gain the most from this work, we will do the following:

- We will share all experiments and results with the scientific community, including both

successful approaches and unsuccessful approaches. This information will be shared by publishing it, presenting at scientific conferences and giving workshops.
- We will share the results of this project with the public. This will allow people who are interested to learn about the research that is being carried out on tissue repair and regeneration.
- We will share results from this work before or at the same time that we send them to be published. This will make sure that the work is available to other scientists sooner and will help other laboratories avoid repeating the same work unnecessarily.
- We will work together with other scientists and/or research and development companies. We will share knowledge, skills and techniques that may result in exciting scientific discoveries.

Species and numbers of animals expected to be used

- Mice: 17332

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 study we are using mice to study tissue regeneration. This is because the mouse digit tip and the human fingertip are very similar. Both digit tips are made of skin, nerves, blood vessels and bones. Both naturally regrow the tip when injured as well. The things that they have in common make the mouse digit tip an excellent replacement to studying regeneration in humans as it makes us confident that any results we get will also be useful for people. Additionally, all the genetic tools that we need to help us understand how regeneration works are already available in mice. Our first experiments will use cells of interest to us taken from baby mice (less than 14 days old) that will be grown in the laboratory. This allows us to reduce the number of animals we need because we can increase the number of cells we have by growing them. Most of the animal experiments will use adult mice (more than 8 weeks old) however as we need to understand why many animals have the ability to regenerate tissues when younger but in adults this ability is limited. By studying this process of regeneration in adult tissue we can ensure that all of our findings will have the widest clinical application.

Typically, what will be done to an animal used in your project?

In order to study digit tip regeneration, animals will normally have a surgical procedure. During this procedure we will do one of two things. We will remove 1-2 mm of the end of the digit, which will fully grow back. In a human this would be the same as removing the tip of your finger starting from midway through your nail. Otherwise, we will remove 3-4 mm of the end of the digit. This type of injury will not grow back but will heal and the mouse will have a shorter digit. In a human this would be the same as removing the tip of your finger up until the first joint. A small number of animals will also be injected with various substances that will help discover at the cellular level what is happening during regeneration. Animals are subsequently humanely killed and tissue samples taken after death.

To study what certain cells are doing during the process of digit tip regrowth, we will make, breed and use genetically modified animals in some experiments. These animals will receive injection of substances which will either make cells in the digit tip glow, delete a gene in cells affecting their function or make a small number of cells die. In all cases, the mice will be killed humanely and the tissue samples taken after death. We are then able to study what effect this had on digit regrowth under the microscope.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the mice used in this project are not expected to display any harmful changes that affect the animal's day to day life. Amputation surgeries will only be performed on the middle three digits of one or both feet in order to make sure the injury does not affect the animal's ability to move and groom itself.
Surgeries will only be carried out on hindlimbs (feet). The forelimbs (hands) of the mice will not be touched. All surgeries are carried out under anaesthesia and animals receive pain medication that will remove any discomfort during the surgery and in the recovery period. Mice normally recover from surgeries within 10 - 15 minutes and display normal behaviour. When baby mice receive surgery, immediately afterwards, they are returned with their brothers and sisters to their mother. The mother has always been observed to welcome her babies back. Mice that had 3-4 mm of the tip of the digit removed, in which the digit doesn't regrow, will display a small amount of swelling in the digit. This appears at 7 - 14 days post amputation and will naturally disappear following this period. This swelling has not been observed to cause the mouse distress or impact their normal behaviour. Genetically altered mice that are given the drug, tamoxifen, may lose up to 10% of their weight. This sometimes occurs during the first 1 - 2 days that they are given tamoxifen. These mice normally gain the weight back to normal in the next 5 days and do not show any other harmful side effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

This project will use mice. 44% of the protocols are classified as mild and 56% of the protocols are classified as moderate.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The digit tip is a complex structure being comprised of many different cell types. When the digit is injured, it needs to be able to tell all these different cells to regrow a toe over

several months. These cells also need to respond to instructions coming from the surrounding cells which tell them where to go and what to do during the regeneration process. It is therefore important to study this process in a whole organism because the response of the different cell types depends on which cells surround them and what instructions they receive from these cells. Today, there are few models that can imitate fingertip or toe regeneration in the laboratory. Those that do, first require the researcher to obtain cells directly from the animal. Additionally, most techniques that work with cells outside of living animals do not fully reflect their normal behaviour over long periods.

Which non-animal alternatives did you consider for use in this project?

In recent years scientists have developed a new way of growing (culturing) cells in 3 dimensions. They allow scientists to carry out their research without using animals. This is because they can be made using human or mouse stem cells. They are also very exciting for researchers to use because the cells mimic the structure of a small organ and can be grown for long periods of time. These cultures are called "organoids". So far, scientists have been able to make "organoids" that resemble the brain, kidney, lung, intestine, stomach and liver.

Why were they not suitable?

Despite the excitement around this new way of growing cells in three dimensions, scientists have not yet developed an "organoid" that models the human digit tip. Current "organoid" cultures cannot be grown together with supporting blood vessels and immune cells (cells that keep our body safe from infection) as well. This technology, therefore, cannot be used to model how the environment communicates with and directs the cells of the digit tip to regenerate.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 by talking with a trained statistician. This is to make sure experiments are designed properly and have also been based on the number of studies that we expect to carry out each year. It is important to make sure that experiments use the proper number of mice so that the results of the experiments are accurate.

We have asked for advice from experienced researchers to help us work out the number of genetically altered mice needed. We have also predicted the number of animals needed from our own past experience. We have carefully considered the best way to make sure we keep the lowest number of different types (strains) of mice for breeding. At the same time, we have also made sure that we have enough mice to use for experiments.

We have considered the type (strain) of mice that we are working with to estimate the number of young animals that we can expect each time they give birth. For example, mice that are brown normally give birth to 6 - 8 animals. However, white mice give birth to 10 - 15 mice normally. Animal numbers will also be reduced because we will be buying in a large number of them. By doing this, we can control how many mice we have and will not waste any extra that are born and cannot be used.

Test experiments, called pilot experiments, 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 won't harm the animal in anyway. This will then allow us to perform the intended experiment using enough mice to be sure that the results we obtain are right.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To work out the number of animals required for each experiment we used the NC3R's Experimental Design Assistant. This makes sure that the number of animals used in this study will produce data that is reliable and accurate. We have also taken the advice received from a local, qualified statistician. This will make sure that each experiment includes methods of reducing the chance that we unfairly interpret our data. We will also use a type of mathematics, called statistics, to help us calculate the chance that our ideas, called a hypothesis, are correct. Where possible, all experiments are designed to get the most information using the least number of animals possible. For example, we can give the digits on one side of the mouse the treatment and digits on the other side of the mouse can be given the control. Additionally, all experiments were 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?

In order to use the smallest number of animals that will give us useful results, we will do the following:

- We will try and breed our genetically modified mice in a way so that none are wasted. Any types (strains) of genetically modified mice that are not being used for scientific study will be frozen as embryos.
- Where possible, we will do test (pilot) experiments. For example, when working with substances that stop cell function, we want to use an amount that will have an effect. These test experiments will help us to work out how much this amount is but will only use a small number of animals. We will only work with substances that we know. These substances will also have been used in the past for experiments and will have been shown to have an effect before when other scientists used them.
- We will try to work with other scientists and share tissue from the mice that we have removed the digits from. This will reduce the need to breed more mice.
- We will keep up to date with any new techniques that allow us to replace the experiments where we use animals with different experiments that do not use these.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use mice to study the naturally occurring process of digit tip regrowth. The procedure of digit tip amputation will be carried out in two different ways. Amputations that only remove 1-2 mm of the tip of the toe will result in regrowth of the digit to its original shape after approximately 30 days.
Alternatively, amputations that remove 3 – 4 mm of the tip of the toe will result in wound healing and scar formation. Additionally, digit tip amputations in mice only affect the skin, the very tip of the bone, minor nerves and nail. This injury does not damage muscles, tendons, glands or joints. Because these amputations only affect a small amount of tissue, the digit is able to heal quickly. It does not affect the ability of the animal to move or it's day to day activities. This project will also use genetically altered mice. Using these animals, we are able to follow cells or kill cells that we are interested in. These genetically altered mice have been used by other researchers before. They are not expected to show any harmful clinical signs.

Why can’t you use animals that are less sentient?

The mouse is a good system for studying naturally occurring regeneration. The process of mouse digit tip regrowth mimics human fingertip regeneration. In this project, most of the time we will be studying adult mice. The reason for this is that many animals have some ability to regenerate when they are young. When the animals reach maturity however, this ability is lost. In mice, the end of the digit tip is able to regenerate throughout the mouse's life. This also occurs in humans and we would like to understand why this is so. Mice were also chosen for this study as this is the only type of animal in which all of the genetic tools needed are available. Regeneration in classes of animals that are less aware (sentient) such as amphibians,, fish fish or worms have been shown to act through different mechanisms to mammals. These animals can produce and use specialised proteins which mammals can't make.

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:

- We will make sure that animals suffer as little as possible during surgical procedures by giving the animals medication to manage their pain. All digit tip amputation surgeries and injections into the digit tip will use anaesthesia. Pain medication will be given before the surgery and where necessary after the surgery. We will talk with the Named Veterinary Surgeon to make sure we do this correctly.
- We will make sure to watch the animals before and after surgery to make sure they are healthy and not distressed. We will record what we see on a chart. If the animal needs anything such as more pain medication, we can give this to them. We will

always talk with a Named Veterinary Surgeon and get advice if the animal shows any signs that are outside its normal behaviour.

- 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 LASTA Guidance on Preparing for and Undertaking Aseptic Surgery (2017).
- 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 with other mice that they get along with. The mice will be given enough bedding material and at least one shelter (e.g. cardboard tube or plastic house). This will encourage the mice to explore and give them a refuge so they can escape the other mice when they want to.
- We will make sure to kill any animals before they suffer too much. This is called a Humane
- Endpoint. We are not expecting animals that undergo any procedure in this licence to suffer very much or for very long. If an animal does begin to look unhealthy, we will look at it and decide if it needs to be killed. If the problems cannot be relieved with pain medication or other methods, the animal will be killed.
- Some substances have to be given to animals by specific routes. We have therefore asked to use several different routes (e.g. intraperitoneal [injection into the abdomen], oral and subcutaneous [injection under the skin]) in this project. We will always use the least harmful route possible to give an animal a substance, which is normally under the skin (subcutaneous). 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 their handlers prior to use. This will reduce the amount of stress the animal experiences and will improve their well-being.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To make sure experiments are done in the best way, we will follow the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. For surgical procedures we will 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_data/file
- /773553/GAA_Framework_Oct_18.pdf https://www.nc3rs.org.uk/GAmice
- Additionally, for making sure that chemical inhibitors or ligands are given in the best way, we will refer to the following resources:
- *"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". Morton DB et al. Lab Anim. 2001 Jan;35(1):1-41*

- *"Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider". Turner PV et al. J Am Assoc Lab Anim Sci. 2011 50(5): 600–613*
- *http://www.procedureswithcare.org.uk/lasa_administration.pdf*

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

In order to stay up to date about advances in the 3Rs, I will use the following methods:

- I will make sure that I have regular talks with the Named Persons and animal technicians at my Institute. This will help me to go over current approaches and remain informed if there are any new 3R opportunities.
- Sign up for the NC3Rs e-newsletter. This is where information on the latest NC3R publications is promoted. I will also attend NC3Rs events or workshops.

- I will regularly use our Institutional 3Rs search tool. This contains a database of information that is frequently updated about ways to reduce or replace the number of animals we use in experiments. It also contains information about the best ways to perform experiments that will reduce stress on the animal.
- I will regularly search published literature to stay informed about the latest techniques and approaches that may be used that will allow me to replace, reduce or refine my experiments that use animals.

To make sure that I can use any new techniques properly, I will collaborate with other scientists or staff members that already know these new techniques. This will make sure that all scientists working under this licence receive proper training and are qualified in the new procedure. Test studies will be performed for any refined methods to make sure these new methods do not cause the animal any more pain, suffering or distress. If any of these new methods occurs in a procedure that requires authorization (regulated procedure), permission will be sought before any experimental work is started.

81. Anthropogenic impacts on migratory fish

Project duration

5 years 0 months

Project purpose

- Basic research
- (e) Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

migratory fish, barriers to migration, life history variation, environmental change, fish pass

Animal types	Life stages
European eel	adult juvenile
Salmonids	adult, juvenile
Marine Species representative pelagic and benthic species native within UK waters, excluding those listed in Annex A to Council Regulation (EC) No 338/97	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 is to, first, identify and quantify key components driving patterns of fish behaviour and movement ecology and, second, define and test measures to mitigate for the impacts of selected anthropogenic activities on fish behaviour, migration and survival.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Migratory fish that need to move between different habitat types to complete their lifecycle are particularly susceptible to the impacts of human-induced environmental change. These include over- fishing and habitat degradation, habitat alteration and loss. Since 1970, populations of migratory freshwater fish have plummeted, with an estimated 76% decline

globally and a 93% decline in Europe. Many migratory species such as Atlantic salmon, sea trout and European eel are both ecologically and commercially important and there are strong legislative drivers to conserve their stocks. Understanding how fish biology and behaviours are affected by environmental changes and how we might mitigate for any negative impacts is urgently needed in the face of ongoing declines.

Addressing barriers to migration by installing fish passes and protection technologies is one measure to help conserve imperilled fish. There remains a lack of effective designs for many species and/or lifestages of fish and robust tests are urgently needed to ensure new pass technologies are fit for purpose and cost-effective. By using telemetry to track fish movements at a biologically relevant scale, this research will inform water managers about how well fish passes and other fish protection measures operate and provide recommendations for design improvements.

Other studies within this programme of work will focus on gaining a better understanding of fish behaviour and movement patterns as they migrate within and between freshwater and marine environments, primarily to identify human impacts. Our work will be used by policy makers, regulatory authorities and fisheries and aquatic habitat managers to inform effective management of freshwater and marine habitats (e.g. defining protected areas, implementing restoration efforts), and conservation actions for fish stocks (e.g. setting fishing quotas, licensing aquaculture activities). While studies are focused within the UK and will derive site-specific benefits, the wider research questions being answered are highly transferable to many other systems throughout these species' ranges.

What outputs do you think you will see at the end of this project?

The anticipated outputs will include:

- Quantitative assessments of the functioning of various designs of fish passes and protection technologies. Our results will help regulatory authorities and fisheries managers to identify the most effective solutions for improving fish passage and protecting fish at structures. Our recommendations for design improvements will be fed back to pass developers and manufacturers so they can more effectively meet the needs of this sector. Findings will also be disseminated as peer-reviewed scientific literature so will contribute more widely to fish passage science, including in other countries.
- Beyond specific fish pass designs, publication in the scientific literature of the findings from our studies of fine-scale eel movement data in relation to the detailed hydrodynamics they encounter in fish passes. These data are urgently needed to advance fish passage and protection science by 1) providing fundamental and robust design criteria, and 2) assist the switch towards a future predictive modelling approach to fish pass assessment and improvement, thus removing the need to conduct tests of every design with live fish
- An improved understanding of the contribution of factors that drive the movement patterns of migratory fish in freshwater and near-shore areas. We will be investigating a wide range of factors, both those intrinsic to the fish (e.g. physiology, genetics), and external (e.g. human activities, predation). This information is needed by local catchment and fisheries managers so they can implement appropriate measures to help reverse the decline in migratory fish stocks. With regards to estuarine and near-shore areas in particular, this information is also urgently required by the national regulatory authorities to inform their policy-making.
- The communication of findings to local, national and international stakeholders, and the

general public, through workshops, meetings, presentations and publications.

Who or what will benefit from these outputs, and how?

Short term benefits – years 1-5

- Robust assessments of novel fish passage and protection technologies to inform imminent spending decisions on how to meet UK legislative obligations and targets
- International collaboration through working groups and non-formal research networks
- Raised awareness of the issues causing declines in migratory fish through stakeholder engagement

Long term benefits: > 5 years

- New and improved designs for fish pass protection and passage technologies
- Improved guidance on the suitability of fish pass protection and passage technologies for certain species/lifestages/sites
- New information for policy makers tasked with managing the impacts of human activities on salmonid and other fish in transitional waters
- Evidence-based predictive tools to optimise policy and management decision making
- Contribution to wider scientific knowledge on the drivers of life-history strategies in salmonids
- Contribution to datasets on salmonid population abundance and size structure in the study systems. Information that is used to generate stock assessments, inform policy and measure the efficacy of management interventions

How will you look to maximise the outputs of this work?

- We collaborate closely with partners in Government, catchment managers, utilities (water & power), Rivers Trusts and Wildlife Trusts, commercial and sports fisheries and fisheries enforcement agencies. These collaborations help inform our research direction to maximise tangible outputs
- We contribute to national and international working groups for the study species. We work directly with Government policy makers and regulators to shape the future direction of management and conservation, particularly within the fields of anguillid and migratory salmonid conservation.

- Our organisation hosts regular conferences, species working groups, and conservation special events. We actively disseminate our work to a wide audience through these channels.
- Even though our work is of greatest importance nationally and therefore is often disseminated through internal reports and stakeholder workshops, we have a strong track record in publishing our findings in the wider peer-reviewed scientific literature. In this way it contributes to a global knowledge base and minimises the risk of others replicating the same work.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The chosen species are of specific interest because they have undergone population declines and are the subject of legislation aimed to bring about stock recovery. The lifestages are selected based on where the need to fill knowledge gaps is most pressing.

Typically, what will be done to an animal used in your project?

Fish will either be tagged and/or marked so that their movements can be tracked. Tagging entails anaesthetising individuals then either making a small incision through which the tag is gently pushed into the peritoneal cavity or attaching it externally using thin wires passed through tissues near the dorsal fin. Some tagged individuals will also be externally marked by inserting a needle close to the dorsal fin and attaching a small T bar anchor tag. This is so they can be easily recognised as tagged fish by 3rd parties who may capture them (e.g. anglers or commercial fishers).

Some individuals will be sampled for blood before they are anaesthetised and tagged. This entails inserting a needle close to the tail of the fish and withdrawing a small quantity of blood (maximum 10% of fish mass blood volume). It is a quick procedure that typically takes less than 3 minutes. Some individuals may be sampled for fin tissue after they are tagged and while they are still anaesthetised. This entails using a sharp pair of scissors to snip off the top section of the adipose fin. Juvenile fish too small to be tagged will be marked using a dyed compound that glows under UV light. For this procedure, the fish is anaesthetised and a small amount of the compound (0.01 - 0.02 ml, fish size dependent) is injected just under the skin using a hypodermic needle.

The maximal duration of all the surgical steps combined will not typically exceed 15 minutes. After surgery, the fish is transferred to a holding cage in the river or an aerated holding tank for recovery. In some studies, fish will be released into the wild after recovery. In other studies, fish will be retained and used in trials to test fish pass designs before being transported and released into the wild.

What are the expected impacts and/or adverse effects for the animals during your project?

A possible adverse effect could be infection of the tagging wound. Infection risk will be minimised by taking adequate infection control measures (e.g. antiseptic precautions) during tag implantation.

Expected severity categories and the 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 classified as either mild or moderate severity.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The study programme is focussed on advancing our understanding of the behaviour of actively migrating fish. There is no appropriate replacement for wild fish because at present there is insufficient existing knowledge on fish behaviour to accurately predict and model the natural movements of wild fish.

Which non-animal alternatives did you consider for use in this project?

An alternative approach we considered to test barrier mitigation strategies (e.g. fish passes) for eels is to use computer modelling approaches such as individual-based models.

Why were they not suitable?

The current state of the science makes them insufficient for this purpose. The best available approaches (e.g. Padgett et al, 2020) fail to capture the complexity of eel movement because eels interchangeably use swimming and climbing during the ascent of structures and models fail to incorporate climbing behaviours. Further, there is currently a dearth of data with which to parametrise the models; Padgett et al. (2020) used burst swim data dating back to 2004 that was based on a very small sample size of eels.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of fish represent the minimum required to obtain statistically robust results, based on the scientific literature, previous research experience and statistical tests.

For controlled tests of barrier mitigation technologies under different treatments (e.g. flow rate, fish pass slope), numbers are derived from power calculations based on treatment effect size informed from our previous work. Specifically, we will mark with visible implant elastomer a maximum of 3100 juvenile eel, which represents 20 individuals per trial. Larger juvenile eel will be PIT tagged. Unlike marked fish, PIT tagging allows for definite individual identification, so the within tank (i.e. between individuals) variance will be more accurately defined, and is likely to be lower than in our previous studies. We calculated that 5 replicates per treatment (i.e. 50 eels) are sufficient to detect the estimated treatment effects above the expected levels of variability/noise. For tests of European eel passage

and protection technologies in the field, we aim for 30 tagged fish per release group, and a minimum of 3 replicates per test condition. Prior experience indicates that this is the minimum required to gain meaningful entrance and passage efficiency metrics, allowing for the fact that a proportion of the fish released may not approach the pass. For the studies of European eel fine scale movements and responses to flow cues in lentic waterbodies, our target number of 60 tagged fish per year is strongly informed by our previous work in a reservoir that employed the same positioning telemetry system, and our previous fine-scale eel tracking in an intake forebay. Across all studies, the maximum total number of eel to be tagged is 6,300.

For studies on the behaviour of free-ranging fish, the telemetry methods being employed maximise the likelihood of gaining high quality and quantity data from each individual, thereby minimising the number that need to be tagged to gain meaningful results. Additional logistical constraints such as the numbers of fish likely to be captured in the study systems, staff time and the cost of tags are also contributory factors in decisions on study design, including tagging numbers. However, past experience and published studies were the primary information used to ensure that the numbers will enable sufficient inference to achieve our objectives.
For the studies of salmonid life history variability, our target is to tag 30 fish per year from each minor river and 60 fish per year from each medium to large waterbody, giving a maximum of 3750 fish. These numbers take into account our anticipated acoustic receiver array efficiency and return rates based on previous studies and should allow us to generate accurate estuary/marine survival rates in addition to movement patterns. For studies of marine species, we will tag a maximum of 800 individuals, comprising 200 individuals per system, over four discrete studies. This, based on the detection rate achieved in previous similar studies, is the minimum required to achieve robust assessment of the impact of anthropogenic pressures in large marine environments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We conducted detailed literature reviews to inform our decisions in situations where we had no relevant previous or pilot studies. Power and sample size calculations were used in the planning stage to ensure our experiments can meet our objectives. Further, we have designed our studies to always optimise the data quality and quantity obtained from each individual through using state of the art methods to achieve our objectives, rather than relying on bulk, low quality information.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tissue samples collected for genetic analysis can be preserved for long periods. Any tissue remaining after our own analysis will be retained for future use by ourselves or fellow researchers in our network, thereby reducing the need to go and sample more wild fish 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.

All proposed fish capture, handling equipment and methods are specifically designed to minimise stress and avoidable harm e.g. adequate through-flow of water, soft knotless nets, appropriate mesh/bar size for species, smooth edges to frames, nets, tanks etc. All nets, traps and equipment will be used by competent staff with prior experience of the proposed methods. The proposed capture and handling equipment and techniques result from extensive testing and refinement by fisheries scientists around the world including UK government science and monitoring agencies. Nets/traps will be checked regularly (minimum every 24h) with captured fish removed immediately and placed in suitable holding facilities. Electrofishing will only be conducted by certificated experienced staff and is deemed an efficient non-harmful method employed for repeated long term fisheries monitoring programmes.

Specific procedures within this project are:

- Tagging with a tracking/monitoring device by insertion into the peritoneal cavity- The surgery required will only be carried out by the principal investigator and co-investigator(s) who have extensive experience using the proposed methods on the proposed species. The incision that will be required to insert the tag will be on the ventral surface in an area chosen to minimise the chance of puncturing any major blood vessels. All tagging apparatus including sutures and tags will be sterilised using appropriate sterilisation fluid and antiseptic precautions will be used throughout the procedure.
- Attachment of an external marker tag - Markers are chosen so that the monofilament or wire section inserted in the fish is of small (<1 mm) diameter, minimising the risk of an adverse tissue reaction to the tag, and tags are of a suitable size relative to the fish so that the effects of additional drag during swimming are minimal. A small number of scales (approximately 3 to 5) will be removed from an area at the point of insertion to reduce impact on surrounding tissues. The tag will be inserted at an acute angle to the body and will be in line with the body when the fish swims, thereby reducing resistance and tag movement. The tag will be placed behind the pterygiophores to reduce the likelihood oftag loss.
- Visible implant elastomer marking - This was chosen because it's among the most effective and minimally invasive ways to mark fish with translucent skin, requiring only one insertion of a narrow gauge hypodermic needle. Replacement of the needle between animals further minimises the small risk of infection at the injection site and reduces tissue trauma because the needle is always very sharp.
- Removal of the adipose fin to obtain tissue - This is the chosen location to sample tissue because it minimises pain as few nocireceptors are located on the fins, and the adipose fin is the smallest fin and is considered functionally redundant in the study species.
- Blood sampling - Blood will be withdrawn from a single needle inserted in the caudal region. The quantity is small and scaled on fish body mass, so smaller fish will not be disproportionally impacted. Antiseptic precautions and replacement of the needle for each fish minimises any risk of infection and reduces tissue trauma because the needle is always very sharp.
- Scale sampling - The removal of scales represents a reliable and minimally invasive

way to age our study fish and provide information on past life-history. Only 3-5 scales will be removed for this purpose and the sampling location, on the dorsal flank, away from the lateral line which has a high concentration of neuromasts, is selected to minimise distress and suffering. The scales will regrow causing no lasting harm.

When fish undergo any procedure with recovery, they will be monitored for an appropriate period to assess any adverse effects and ensure minimum suffering. It is the most likely outcome that fish subjected to the procedures and released to the wild will suffer no long-term impairment.

Why can’t you use animals that are less sentient?

The species and lifestages of fish chosen are of specific interest so they cannot be replaced with other lifestages. We are capturing wild fish and none of the experiments require the fish to be killed, therefore terminal anaesthesia is not appropriate both because we don't want to deplete wild stocks and because the data we hope to gain from the fish relies on tracking their behaviour post-procedure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our proposed procedures represent the most refined methods based on current knowledge, and we actively keep up to date with advancements in the field.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We keep up to date with animal welfare literature and practices. Our fish capture and transport methods follow Environment Agency best practice guidance. All activities involving the housing and husbandry of animal within our institute are reviewed and refined by veterinary staff.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am part of a global network of researchers who conduct similar research and through collaborative studies and workshops we actively exchange ideas and learn from each other's approaches. For example, we share developments and findings through specialist groups such as the Oceans Tracking Network, European Tracking Network, International Conference on Fish Telemetry, and the International Bio-logging Science Symposium. We, as a community, always strive to reduce and refine our use of animals. Rapid advancements in tracking technologies and non-fish surrogates (e.g. robot fish) offer the most promise for achieving this. Staying informed about the opportunities to use computer modelling in place of, or in combination with, animal experiments is also something I and my team actively do. At my own research institute, I am a member of the 'Biotelemetry: advancements in technology and methods' review group, which includes both researchers and veterinarians.

82. Transcriptional regulation of erythropoiesis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

red blood cells, anaemia, transcription factors, leukaemia, proteomics

Animal types	Life stages
Mice	adult, embryo, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the process of generating red blood cells in our body is controlled and how these control mechanisms can go awry in blood diseases, such as anaemia. We will focus on the study of a protein called GATA1, which is a critical player controlling the process of red blood cell generation.
Importantly, mutations affecting GATA1 functions have been linked to blood disease, including leukaemia.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 us to understand basic control mechanisms in the generation of red blood cells in our body. This is important because it will provide us with new knowledge that will help us understand how these control mechanisms go awry in diseases such as anaemias (sickle cell anaemia, thalassemia etc.), porphyrias and leukaemia. Specifically,

the project will involve the use of genetically modified mice to study the fundamentals of GATA1 function, particularly during blood cell generation in the foetus. This is important as it has been shown that specific GATA1 mutations are strongly associated with anaemia in the foetus, which can be fatal, and with leukaemia in Down syndrome babies. Studying GATA1 in the blood cell generation in the foetus cannot be modelled or recapitulated in simpler animal models or in cellular models.

What outputs do you think you will see at the end of this project?

The work to be performed will provide new information on the role of GATA1 in red blood cell production in our body and on how mutations in GATA1 result in blood diseases. This information may lead to the development of new therapeutic approaches in the treatment of haematological disease such as anaemia. Our work will also result in the development of novel methodology for analysing proteins, that we expect will have many applications and which may lead to commercial products and/or services. We also expect our work to be disseminated through presentations at international conferences and through scientific publications in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

The red cell and haematology research communities will benefit initially from the outputs of this work. In addition, the outputs of our work will go some way in helping us understand the involvement of GATA1 mutations in haematological disease. In the long term, this will help in the development of effective treatments. GATA1 belongs to the GATA family of proteins which includes a total of 6 members with important roles in different organs, including the immune system, the heart, the pancreas and others. Mutations affecting other GATA proteins have also been implicated in disease, for example, GATA2 in leukaemia and GATA3 in breast cancer. As such, the outputs of the work described here will also broadly benefit research on GATA proteins and, in the longer term, will help develop more effective therapies for GATA protein-associated disease.

How will you look to maximise the outputs of this work?

We will collaborate with researchers in Germany for the proteomic characterization of GATA1 protein functions and with researchers at our University for the analysis of genomic data, which will identify the genes that GATA1 controls in red blood cells. Our work will be presented at conferences (American Society of Hematology Congress, GATA Meeting, Haemoglobin Switching meeting etc.) and published in relevant journals. Data will be made available by depositing in appropriate public repositories.
Detailed protocols and unsuccessful approaches will also be submitted for publication to inform the broader research community.

Species and numbers of animals expected to be used

- Mice: 8500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use different genetically modified mouse models that will allow us to directly examine the functions of GATA1 in red blood cell generation and of GATA1short, i.e. a GATA1 mutation that has been associated with leukaemia in Down Syndrome children and with the rare haematological disorder Diamond-Blackfan Anaemia. The mice have been genetically modified through the addition to the GATA1 protein of a small “handle” that will allow us to efficiently and cleanly purify GATA1 and GATA1short proteins directly from red blood cells in order to characterize their functions. As the haematological disorders associated with the GATA1 mutations (i.e., GATA1short) first appear in the foetus, our work will focus mostly on the collection of embryos at specific stages of gestation that correspond to the foetal stages that are relevant to our key scientific questions.

Typically, what will be done to an animal used in your project?

Animals in our project will be primarily used for breeding, blood sampling and collection of embryonic tissues. Mice will be ear clipped for identification and genotyping. Successful breeding pairs will stay together for no more than 4 breeding cycles. Females used in timed breeding will be killed humanely. This is necessary in order to retrieve embryos at specific developmental stages (embryonic day 11.5- 14.5). Use of general anaesthesia is not recommended as it can affect the physiology of the embryos and the experimental outcomes of the study. Male and female mice will be used for tail vein blood sampling.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect any adverse impacts or effects for the animals during the project. We will only conduct minimally invasive procedures where the procedure occurs without terminal anaesthetic, such as ear clipping and blood sampling. The expected impact of ear clipping and blood sampling is expected to be minimal. Aseptic technique will be used to minimise risk of adverse effects following blood sampling. Analgesic cream will be applied to site of ear clipping and blood sampling, when required. Even though no adverse effects are expected from these procedures, mice will be monitored for 24h following sampling. Animals exhibiting any unexpected harmful phenotypes will be killed humanely. Embryonic tissue will be collected from mice following schedule 1 protocols and are therefore non-recovery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: tissue biopsy for genotyping mice in breeding; also for blood sampling. Female mice will be culled by a humane method for embryo collection.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to study the physiological functions of the GATA1 protein in red blood development in the foetus and how these functions are disrupted by the GATA1short mutation. We need to use mice to study the early window in the development of the blood system in the foetus, which appears to be particularly susceptible to GATA1 dysfunction. As this work involves mammalian foetal developmental, it cannot be carried out using lower vertebrates (e.g. zebrafish) or cultured cells as surrogate models.

Which non-animal alternatives did you consider for use in this project?

Alternative non-animal models considered include cultured red blood cell types derived from leukaemic samples, or from the differentiation in a petri dish of embryonic stem cells or of induced pluripotent stem cells. All methods to be used in the project will initially be carried out and optimised using cellular models so as not to use animals unnecessarily.

Why were they not suitable?

Working with red blood cells in a petri dish cannot recapitulate the events taking place during the generation of red blood cells in the mammalian foetus. The sites of red blood cell production change as foetal development progresses. The red blood cells generated as these sites shift, differ in their properties so as to better suit the specific demands of the growing foetus. In addition, the niche, i.e. the cells surrounding the changing sites of red blood cell generation in the foetus, play a critical role. All these parameters cannot be recapitulated in any of the existing cellular models for red blood cell 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?
We have estimated the total number of mice based on:

the number of mice we will need to crossbreed to homozygosity and to maintain the different genetically modified strains used in the project. We will need to maintain colonies for the following transgenic lines (six in total):

Homozygous BioGATA1/ROSA26-BirA double transgenics

Homozygous BioGATA1/UCOE-BirA double transgenics

Homozygous BioGATA1short/ROSA26-BirA double transgenics

Homozygous BioGATA1short/UCOE-BirA double transgenics

Homozygous ROSA26-BirA single transgenics

Homozygous UCOE-BirA single transgenics

We will need to maintain for each homozygous line a minimum of 4 males and 6 females (60 mice per breeding cycle for all lines) that will be used to increase colony stock as required for experimentation, assuming that homozygous transgenic lines will be replenished every 4 months (3 breeding cycles per annum). Taking also into account the number of crosses that will be required to obtain the homozygous double transgenic lines, we estimate for this part of the project that we will need a total of 7500 mice for the five-year duration.

the number of mice we will need to carry out our experiments using mouse embryos. For our studies we will need to collect embryos between developmental stages E11.5 and 14.5 using the different transgenic mouse strains given above. In estimating the number of animals we will use for embryo collection, we took into account an average litter size of 8 embryos, the amounts of experimental material that we will obtain at each embryonic stage and the need to carry out all experimental assays in quadruplicates for reproducibility. Taking all these factors into account, we estimate a total of 800 mice for this part of the project.

The number of adult transgenic mice that we will need to obtain blood samples from the tail vein. We estimate that we will need a total of 200 mice for this part of the project.

Grand total: 7500+800+200 = 8500 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The main determinant of which animals and how many animals are used in this project is the genotype of embryos from which we will obtain tissues for analysis. Heterozygote and homozygote breeding pairs will be used for timed pregnancy experiments to (i) initially collect all required samples for analysis including controls and to subsequently maximise the generation of embryos with the specific genotypes required and avoid wastage of females and embryonic tissue. Embryonic genotyping requires the humane killing of pregnant females. Experimental data are also restricted by the number of cells available in specific tissues at specific developmental stages (i.e. earlier developmental time points will yield less starting material). Improvements in experimental technique and design have maximised the amount and improved the quality of data obtained from a reduced number of cells, thereby reducing the number of mice required for experiments. Lines will be frozen down and re-derived as required to restrict maintenance breeding. Maintaining lines as heterozygotes and homozygotes only will reduce the number of non-genetically modified mice generated.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals will only be used when absolutely necessary. In order to minimise the number of mice used in this project, we will breed heterozygous mice which will generate all required controls for the analysis (i.e. wild type mice, single transgenics [BirA or bioGATA1/bioGATA1 short] and double transgenics).
Initial proteomic and genomic analyses of the control samples will generate sufficient reference data for subsequent experiments. This will allow use of homozygous double transgenic lines to collect additional samples, thus reducing the number of animals used

per experiment. Once double transgenic lines have been established, we will look to cryo-preserve the single transgenic lines to reduce the number of mice we maintain for the project. Whenever scientifically feasible, we will seek to switch to using cell lines to further test, validate and extend the data obtained using mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically modified mouse models. The mice carrying these modifications already exist and are viable with no adverse phenotype or lasting harm. We will isolate embryonic tissues at specific developmental stages (E11.5-14.5) that coincide with the transition from primitive to definitive erythropoiesis. Embryos will be isolated at developmental stages prior to the last third of gestation.
Pregnant females will be killed under a schedule 1 method. In rare instances, it will also be required to collect bone marrow. Again animals will be killed under a schedule 1 method. These methods are not expected to cause pain or suffering, distress or lasting harm.

Why can’t you use animals that are less sentient?

We will be using primarily embryos up to 14 days of gestation. Zebrafish and lower vertebrates are also used for developmental hematopoiesis studies, however a mammalian model still remains necessary to provide key insight and understanding of the disease-associated phenotypes that form part of this project and of their physiological effects in complex developmentally regulated processes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Best practice will be used for all procedures. All cages will be enriched with nesting materials. Daily non-intrusive monitoring of all animals will be performed by BSU staff and/or scientific researchers. Animals will also be observed through handling in order to train the animals in this procedure. Animals undergoing a terminal procedure (e.g. schedule 1 and embryo collection) will do so away from the sight and sound of other animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will closely follow the best practice guidance of ASPA. We will regularly consult the RVC and NC3Rs websites and look to our AWERBs and NACWOs for current best practice guidance.

How will you stay informed about advances in the 3Rs, and implement these

advances effectively, during the project?

Best practice will be used for all procedures. All cages will be enriched with nesting materials. Daily non-intrusive monitoring of all animals will be performed by BSU staff and/or scientific researchers. Animals will also be observed through handling to train the animals in this procedure. Animals undergoing a terminal procedure (e.g. schedule 1 and embryo collection) will do so away from the sight and sound of other animals.

83. The role of CSF1R in rodent macrophage biology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- 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

Macrophage, Microglia, Neurodegeneration, Development, Disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Increase knowledge of macrophage biology in health and disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

This project is focused on expanding our understanding of the role of macrophages (a cell of the immune system) in immune function, growth and development, as well as health

and disease. It is becoming increasingly clear that macrophages are influential in many physiological processes and diseases. Therefore, this is a significant area of scientific study that will contribute to our understanding of basic physiology and may lead to the development of new therapies important for human health.

What outputs do you think you will see at the end of this project?

We will achieve a greater understanding of macrophage biology in development and disease.

This is important because macrophages are found in all tissues of the body. They are essential for normal development but are also involved in many diseases such as cancer, arthritis, osteoporosis and white matter diseases of the brain.

By the end of this project we hope to identify new drugs that can be used to treat or prevent osteoporosis.

We also aim to explain why mutations in a macrophage-specific gene cause the neurological disease Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP).

We will publish all of our results in open access journals.

Who or what will benefit from these outputs, and how?

In the short-term, our experimental outputs will inform other researchers and will likely result in the establishment of new collaborations so that common scientific goals can be tackled more effectively.

A deeper understanding of the role of macrophages in development and disease may provide insight into developing novel treatments/preventative measures for a range of macrophage-related pathologies.

In particular, we hope to provide osteoporosis patients with a new treatment and provide patients with Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) more information about what causes the disease.

How will you look to maximise the outputs of this work?

All of our research is performed in partnership with scientists who are experts in their particular field. We will disseminate new knowledge with our collaborators worldwide via meetings and publications.

Species and numbers of animals expected to be used

- Mice: 6200 (including colony maintenance)
- Rats: 6200 (including colony maintenance)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 freshly isolated macrophages from humanely killed animals to investigate macrophage properties in vitro and we will continue to use this approach wherever possible and scientifically valid. However, in vitro assays cannot adequately model the complete role of macrophages during development, inflammation or disease as macrophages are just one of a massive number of cell types involved in inflammatory responses and immunity. Therefore, in vivo work is also required to understand how macrophages function in their proper environment.

We use animals at all life stages as macrophages play important roles throughout much of development, adulthood and ageing.

Typically, what will be done to an animal used in your project?

The majority of our experiments involve investigating macrophages from humanely killed animals. For our other experiments, animals are typically:
injected with a compound and blood sample/s taken (range 1 day to 6 weeks). OR
injected with a compound prior to infection (range 1 - 5 days). OR injected with donor cells blood sample/s taken (range 1 - 12 weeks).

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect any adverse effects for the majority of experiments as all the compounds/cells have been used extensively in our laboratory and others.

Some of our genetically altered rodents have adverse phenotypes. These include osteopetrosis (thicker bones) and the absence of teeth. These animals are fed a liquid diet and housed with as few animals are possible to prevent any injuries to their (more fragile) bones. These animals are typically analysed whilst young and are not aged.

We use behavioural tests to determine the role of macrophages in diseases such as Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP).

One of our experiments will involve surgery on pregnant rodents (under general anaesthetic). This may result in mild discomfort whilst the incision heals and animals will be given pain relief.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold 75% - The majority of our experiments involve investigating macrophages from humanely killed animals

Mild 20% - A mild severity is expected where animals are injected and subsequent blood samples taken.

Moderate 5% - A small percentage of our GA rodents have a harmful phenotype due to

the absence of macrophages.

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 use freshly isolated macrophages from humanely killed animals to investigate macrophage properties in vitro and we will continue to use this approach wherever possible and scientifically valid.

However, in vitro assays cannot adequately model the complete role of macrophages during development or inflammation as macrophages are just one of a massive number of cell types involved in inflammatory responses and immunity. Therefore, in vivo work is also required to understand how macrophages function in their proper environment.

Which non-animal alternatives did you consider for use in this project?

We have previously used cell-lines in the majority of our experiments. The promising results obtained have led to this application for in vivo work.

Why were they not suitable?

The non-animal alternatives were suitable for the initial studies. However, macrophages functions are defined by environment-specific factors, such as other cells and local signalling molecules in every tissue of the body. These environments cannot be accurately modelled using non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers were estimated based on our previous 10 years 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.

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 the numbers of animals used by:

- performing pilot studies when required
- breeding efficiently to ensure we have control and genetically modified animals for each experiment
- storing all organs from killed animals following an experiment - for use by ourselves at a later date or to provide organs to other researchers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use genetically modified mice and rats in order to study a particular gene which we know is important for macrophage development. Most of these animals do not have any harmful symptoms. Analysis of these rodents includes behavioural tests to see how the absence of macrophages affect learning and memory. These tests do not cause any suffering or distress.

One of our genetically modified rat lines develops thickening of the bones (due to the absence of macrophages). These animals are monitored daily and housed with as few animals as possible to prevent injury.

The majority of our experiments involve injection of substances which do not have any adverse effects. We do this to either increase or decrease the numbers of macrophages in wild type rodents. For example, we want to determine if reducing macrophage numbers can prevent age-related bone loss (osteoporosis) and if increasing macrophage numbers can prevent neonatal infections.

We will use a neonatal rat infection model to see if increasing the maturity of the immune system (by increasing macrophage numbers) can prevent serious infection. These are very short-term experiments (<5 days) and we will analyse the rats at the very earliest stages of infection before clinical symptoms appear.

Why can’t you use animals that are less sentient?

Our studies do involve animals that are less sentient - for example we use embryonic rodents or rodents under terminal anaesthetic wherever possible.

We cannot use lower organisms such as zebrafish as they contain two copies of the gene we study. This means that they are not similar to humans. They also lack a specific region

of the gene which we know is important for macrophage development in higher organisms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At the beginning, researchers will frequently be exposed to the animals so they can learn the differences between normal and abnormal behaviour and appearance. Animals will be provided an enriched environment and they will be handled frequently to reduce their stress. When animals are required to perform a specific task, they will be familiarised with the new environment before the test. If surgical procedures are performed, we will do regular checks to assess recovery and pain. If there are signs of pain, analgesics will be provided.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

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 of the latest news by consulting resources such as: ARRIVE and PREPARE guidelines, https://www.nc3rs.org.uk/, https://www.gov.uk/research-and-testing-using-animals, www.understandinganimalresearch.org.uk, www.frame.org.uk and http://www.procedureswithcare.org.uk.

We also receive regular updates and advice from our named vets and the Home Office.

We implement these updates via regular evaluation of our study request forms by the named vets.

84. Understanding the mechanisms and pathophysiology of heart failure and atrial fibrillation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (ii) 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

heart failure, myocardial infarction, atrial fibrillation, therapy, comorbidities

Animal types	Life stages
Sheep	adult, aged, juvenile, neonate
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?

Diseases of the heart including heart failure, myocardial infarction and atrial fibrillation are major causes of premature death and ill health. Many people with these diseases are elderly and also have a variety of other conditions including high blood pressure and obesity which are thought to contribute to disease onset and progression. However, the underlying mechanisms causing these associations are poorly defined. This project will firstly investigate how these various factors act to cause these cardiovascular diseases and, secondly, investigate how novel treatments might work in these diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

- Diseases of the heart lead to ill health and premature mortality.
- There are no fully effective treatments for these diseases with five-year survival for key heart disease in Western society being worse than that of the commonest cancers e.g breast and prostate cancer.
- A key factor for the lack of progress in finding effective treatments and preventative measures for some of these cardiac diseases remains a lack of understanding of the underlying mechanisms responsible for their development.
- We know that ageing, high blood pressure and being overweight are associated with the development of many cardiovascular diseases but the mechanisms by which these cofactors act to increase disease prevalence are poorly understood.

To address these issues we will investigate key mechanisms that cause the development of heart disease and why heart disease leads to an increased risk of death. We will study the impact of heart disease on the function of the heart at both the whole animal and isolated cardiac muscle cell levels.

From these studies we will gain a better understanding of the mechanisms that lead to various cardiac diseases (the pathophysiology of disease) and from these findings we expect to reveal novel therapeutic targets.

What outputs do you think you will see at the end of this project?

The major output anticipated from the programme of work is an increase in our understanding of the pathophysiology (disease mechanisms) of key cardiac diseases and the role of significant cofactors (ageing, obesity and high blood pressure) in the development of heart disease.

We will also build on our recent findings, by furthering mechanistic understanding, where we reported that a class of drugs normally used to treat erectile dysfunction were highly effective at treating heart failure and reversing some of the changes that occur in the diseased heart.

From the increased understanding of the pathophysiology of heart disease and the role of key cofactors in the development and progression of these diseases, we anticipate that we may be able to develop more effective therapies for heart disease.

We will generate 'large' datasets from experiments where changes in protein abundance and gene expression are investigated.

From these studies we anticipate identification of new and more effective therapies for heart disease.

Who or what will benefit from these outputs, and how?

The major beneficiaries of these outputs in the immediate term (lifetime of project) will be within academia and the pharmaceutical industry.

The increased mechanistic understanding we generate will drive further investigational studies by the wider scientific community and pharmaceutical industry. We anticipate that these 'secondary' studies will commence once our initial findings become publicly available via publications and conference presentations (3 - 7 years).

We would reasonably expect that a successful outcome of our pharmacological interventions could, within a 5 - 10 year period lead to in-human trials. Should these preliminary in-human studies show benefit, the next expectation would be for large scale clinical trials and ultimate adoption of the therapies into standard clinical practice (beyond 10 years).

How will you look to maximise the outputs of this work?

We will maximise accessibility of outputs via open access publishing and depositing large datasets in public databases. This will allow other individuals and groups to use the available information and datasets with minimal delay and thus to greatest effect.

Any software for analytical work that is developed for this programme of work will be made available either through the journal associated with the data or open access sites such as GitHub upon publication

We will also communicate work (subject to intellectual property considerations) as widely as possible via scientific meetings and interactions with commercial partners via workshops and in response to calls for potential collaborations.

We also host a series of public (schools and adults) and patient engagement events within the host institution and contribute to these routinely to maximise the public understanding of our work. We will look to maximise the reach of this work by establishing an online web resource for patients and members of the public as well as for sharing our scientific discoveries with the wider academic and industrial communities.

We are committed to sharing best practice via open access publications. In the event that studies are unsuccessful our intention is that this information is also made available through appropriate channels including appropriate publications and open access repositories.

Species and numbers of animals expected to be used

- Sheep: 1430
- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

The principal animal model used is the sheep with mice used in some experiments. The sheep is a highly translationally relevant animal model for understanding disease processes in the cardiovascular system. The sheep heart is more similar to the human heart than is the case for species such as the rat, mouse or zebrafish. These similarities include structure, size, heart rate, response to various stimuli and stresses and the way in which the parts of the heart are supplied with blood. An important consideration for our studies is that we are able to employ standard clinical practices and devices to best reproduce what would be undertaken in patients. Due to availability arising from standard farm animal practices and potential welfare issues centred around single versus group

housing, studies using sheep will only be performed using female animals.

Our experiments will mainly be conducted using fully grown adult animals. In a few experiments we will use aged animals and a small number of younger animals. The use of fully grown adult animals means that we are reducing confounding factors such as growth which is a neglected consideration in many other studies. In experiments where we will use aged animals, this is because in some experiments we are studying the role of cofactors in disease and age is a one such key factor. Similarly, in some experiments we will use young animals, this is because in the young animal heart there are still processes occurring such as changes in cellular structure which we may wish to validate and target as a therapeutic approach in the disease model and therefore the young animal model may help inform these choices.

Where mice are used, both genders will be studied. The reason for using the mouse in some experiments is that we will be able to take advantage of genetic modifications. This will allow us to understand the role of specific genes and their protein products where they have either been knocked out or over expressed. In all cases, we will use adult mice.

Typically, what will be done to an animal used in your project?

The typical experiment where animals have a surgical intervention will involve a number of non- invasive assessments of heart function in conscious gently restrained animals where we will measure their electrocardiogram (ECG), blood pressure and heart function by echocardiography (ultrasound measurements of heart function). We may also take blood samples during these procedures either by direct insertion of a needle into a vein or from a catheter that has been placed in the vein.

The majority of animals will then undergo a surgical procedure where we implant a cardiac pacemaker and pacing leads into the chambers of the heart in the same way that is used for patients who have a pacemaker inserted. In some of these animals this will be combined with either using an angioplasty balloon placed in one of the arteries in the heart to induce a myocardial infarction (heart attack). These procedures may also be combined with measurements of the electrical activity of the heart either using the implanted cardiac pacemaker or by leads that are temporarily inserted into the chambers of the heart using the same vessels into which the cardiac pacing leads are inserted or additional devices.

Typically only one device approximately the size of a small box of matches will be implanted however, in a small number of animals a maximum of four devices (normally less than half the size of a box of matches) may be implanted to remotely monitor cardiac function (e.g. heart rate or blood pressure), activity or, to stimulate the vagus nerve in the neck. We will also take several blood samples during the anaesthesia period.

In a small number of animals where a vagal nerve stimulator is used, when this is activated we expect it will lead to a periodic cough and change in vocalisation when the nerve is actively being stimulated (a few seconds every few minutes). These changes will resolve spontaneously over the course of a few days and have no long-lasting effect.

In studies of heart failure we will, following recovery from surgery and a period of monitoring the animal to ensure full recovery from the surgical procedure, activate the cardiac pacemakers to cause the heart to beat faster and induce heart failure. Some of these animals will receive treatments (typically a tablet given orally every day) and some will also have a small blood sample taken weekly.

We will monitor the animals for signs of heart failure and once this becomes evident they will be terminally anaesthetised and a final set of measurements of the electrical activity of the heart made before the animal is killed and tissues harvested for a series of in vitro experiments.

The maximum duration over which any animal will be monitored following its first surgical intervention will be six months.

However, not all animals (approximately two-thirds) will undergo a surgical procedure. Here, animals will only be give a single injection of heparin to prevent blood clotting a few minutes before they are killed for tissue harvest for in vitro experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect animals to make a full recovery from surgical interventions with recovery from anaesthesia and do not anticipate anything more than transient and mild discomfort from procedures performed on conscious animals.

In those experiments where we are studying the mechanisms that are altered in heart failure we do expect animals to develop signs of heart failure which can include increased breathing effort, coughing or tiredness. We monitor animals carefully for the development of these signs and they are humanely killed (as the end of the experiment) once these signs become evident and impact on normal behaviour.

In some experiments we may expect animals to develop abnormal heart rhythms in the main chambers (ventricles) of the heart; the large majority of these will be experienced in anaesthetised animals.
However, there is a small possibility that these abnormal heart rhythms may develop spontaneously in conscious animals. If these are significant, we might expect the animal to rapidly lose consciousness and as such do not expect them to cause pain.
Beyond this expected impacts, there is the potential that a small number of animals could develop any of a range of adverse effects as a result of the procedures that have been performed. These could (they are not routinely expected) include; loss of blood during surgery or from a dislodged cannula, sudden death due to abnormal cardiac rhythm disturbances or adverse reactions to administration of investigational compounds. Animals are carefully monitored by experienced care staff and investigators such that should any of these unexpected adverse effects be experienced by the animal then it is for the shortest duration 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)?

Where animals undergo a surgical intervention with recovery from anaesthesia we expect all of those animals to be within the moderate severity category. Conversely, for many animals the only intervention they will experience will be a single injection of heparin to prevent blood clotting a few minutes before they are humanely killed for subsequent in vitro experiments on the harvested tissues. In these animals there will be no more than

minor transient discomfort and the overall severity limit will be mild.

Overall we anticipate approximately 70% of animals will therefore undergo mild severity experiments and the remaining 30% moderate severity 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 need to use whole living organisms because, whilst heart failure and atrial fibrillation are conditions that originate in the heart they are also influenced by the way that other parts of the body such as the kidneys, lungs and brain behave. Critically, the response of these other parts of the body can then also modify the way the heart behaves and therefore the overall progression of the disease. This cross-talk between the heart and the other parts of the body is complicated and not easy to predict as well as also changing quite rapidly. It is not yet possible to predict the outcomes of these interactions using either computer models or cells grown in a dish.

Additionally, some forms of heart failure are also more likely to occur if you have other underlying conditions such as being elderly, having high blood pressure or being overweight. Again these complex interactions and dependencies cannot yet be faithfully reproduced using non-animal alternatives such as computer models or cells maintained in culture dish conditions.

Our first major aim is to understand the mechanisms which cause the heart to stop functioning properly in diseases such as heart failure, atrial fibrillation or after a heart attack. Our second major aim is to also understand how factors such as age, high blood pressure or being over-weight lead to the onset of heart failure. In each of these aims, we hypothesise that changes in the way that the cells of the heart regulate their calcium levels is a major factor behind disease progression. Based on the findings of these initial studies and from some of our previous work we will also investigate the effectiveness of some new treatments for heart disease in these studies. Again, understanding how these new treatments work requires us to study them using approaches that are both reliable and reproducible where all the complex relationships between the heart and other tissues in the body are functioning.

Given these considerations, we need to undertake our experiments in appropriate animal models that allow us to address our key aims, advance our understanding of heart disease and to identify and test new treatments.

Which non-animal alternatives did you consider for use in this project?

There are several possible non-animal alternatives that we have considered. These include:

- human tissues obtained from either deceased people or those undergoing heart transplants
- cells grown in the laboratory
- computer models

Although there are significant limitations with each of these that prevent us from using them to fully replace the use of animal models, it should be noted that we will use some of these approaches to address some very specific questions where it is not necessary to use an animal and thus we strive to replace the use of animals with these methods as much as is practicably possible.

Why were they not suitable?

In each case there are major limitations to using these non-animal alternatives and these are considered below:

Human tissues

These would mainly be available from hearts removed from patients undergoing heart transplant operations or donor hearts that were not considered suitable for use in the transplant programme. The main issues with using human tissues are:

- Lack of availability - There is a major need for more donor hearts to match the number of patients on the transplant list. This means that, particularly healthy human hearts, are very rare and secondly there is a limited number of diseased hearts for use. If we had to rely on these then we would be very unlikely to be able to complete more than a small fraction of our proposed studies.
- Variability - A major concern in using human hearts is that they are being taken from a very diverse population. For example, important factors such as age, obesity, blood pressure and medical treatments they are taking are highly varied and thus difficult to control for in our experiments. This is a significant concern which both limits our ability to draw firm conclusions and our ability to perform reproducible and reliable experiments that others can follow and work from.

Cells grown in the laboratory - There are several types of cells that could be used including those that were originally obtained from animal hearts or stem cells that have been treated to behave like heart cells. In each case there are number of major limitations to using these cells to answer our questions and prevent us from achieving our aims. The major limitations are:

- Cells grown in a dish are normally of a single type, for example HL-1 cells. This does not even remotely reflect the situation in the heart where lots of different cell types co-exist and interact with one another.

- Cells grown in a dish, even stem cells that have been treated to behave like heart cells, lack the structure of a normal heart cell. This is important because many key processes in the cells of the heart occur precisely because of the structure of the cell. If this structure is not reflected by the cells in a dish then it is unlikely that the response of the cells to a treatment will be the same as would occur in the heart.

Computer models - Whilst these are improving all the time:

- They still lack the ability to accurately reproduce what is happening in the heart.

- Computer models cannot reproduce the complex interactions that occur in disease between the heart and other organs

- Given these interactions between the different cells in the heart and the heart and other tissues, computer models lack the ability to accurately predict how new drugs may work in the intact animal (or human)

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

At each stage of the programme of work we will estimate the number of animals that are required to complete the set of experiments before the series of experiments are started.

To calculate these numbers we use information from our own earlier experiments or those that have been published in the scientific literature or a combination of both of these to determine how variable this information tends to be. From this variability we can use statistical tests to determine how many samples are needed to detect differences between groups of animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have, and our intention is to continue to, consulted with an external statistical advisor who is independent of this programme of work in order to minimise the numbers of animals that are required to address our experimental aims. We also utilise this advice in parallel with information that we have obtained as part of our consideration of key aspects of reproducibility in research as part of our regular laboratory meetings between the various groups that make use of animals in this programme of work.
Tools such as the NC3Rs experimental design assistant are a useful adjunct to these processes and help guide considerations around the allocation of animals to groups.

All of the principal investigators leading their own groups which utilise the animal models in this programme of work are also members of different journal editorial boards and regular reviewers for major UK and international scientific funding agencies. As part of these roles, we are always asked to consider experimental design which reinforces our own thinking when planning our experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For the work involving sheep there is no need to consider efficient in house breeding as animals are purchased from external suppliers as and when they are needed to complete our experimental protocols. Where mice are used, we will aim to purchase animals rather

than breed them specifically. However, where we need to breed animals we will maintain colonies as efficiently as possible and, where possible, use littermates as controls rather than separately bred animals.

In the present programme of work, we do not envisage that we will routinely be performing pilot studies. However, if at any point we do use pilot studies, these will also be considered with a view as to the most appropriate way in which they can also be used to contribute to the final experimental data in order to both inform the number of animals required and reduce animal usage.

The programme of work supports a number of principal investigators and their research groups. Importantly, we share a combined laboratory meeting programme and plan our experiments collectively. This enables us to ensure that when experimental animals are available, they are used by the full range of groups and we do not need to repeat a particular experiment twice for different laboratories.

Where relevant we will also use cell culture experiments or computer models to complement or even replace animal-based research. These are important considerations when we plan our experiments and will be used to, for example, validate test substances or test potential pathways prior to investigating their effect in the intact animal or disease model

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 animal model is the sheep which has been chosen because its cardiac function and response to interventions very closely resembles that in man.

We will use a number of models which closely resemble common cardiac diseases of man (heart failure, atrial fibrillation, myocardial infarction). All of our approaches are designed to be as minimally invasive as is possible (which would generally not be achievable in smaller species) and accurately reflect the same procedures that would be performed in patients requiring only a small skin incision to enable pacemakers to be implanted.

All personnel involved in conducting animal experiments are fully trained in the theory and technical aspects of procedures and only take responsibility for completing these procedures as lead operator once they have worked closely with and been observed by experienced operators.

We also work closely with the named personnel and animal care staff to ensure that effective post- operative monitoring is in place and any remedial actions are effectively applied with minimal delays.

Why can’t you use animals that are less sentient?

To deliver the most translationally relevant data from our studies and elucidate potential new treatments that are most likely to be applicable to man requires that we use appropriate animal models. To achieve this a key consideration is that potential confounding factors such as growth are removed from our experimental procedures. Another important consideration with immature animals is that their hearts are not fully developed and are structurally different to that in adults. As such, key signalling pathways which are heavily influenced by cellular architecture do not function the same way in immature animals as in adults. Thus, the translational relevance of using immature forms is a significant limitation in most of our experiments. Given these considerations, for the vast majority of our experiments, we therefore use adult animals that are fully grown. Similarly, one of our experimental objectives is to understand how certain risk factors for heart failure interact; this requires that we use a model where these key factors such as ageing can be studied reliably. These considerations apply to the vast majority of our experiments; however, in some cases the lack of (e.g.) cellular ultrastructural development in immature animals is a useful tool with which we can address some very specific experimental aims.

Given these important considerations, and our aim to use minimally invasive surgical procedures requiring only a small skin incision, our preferred animal model is the sheep. Rodent models, immature life stages or (e.g.) zebrafish models are unable to address these key considerations and are in many respects fundamentally different to the human heart such that they are not appropriate to address our aims and objectives.

In many of our experiments we are using a disease model. In each case and in humans, the diseases that we are replicating takes a considerable time to develop following the initiating event. This therefore precludes us from completing all aspects of our experiments in terminally anaesthetised animals.
However, we do strive to use terminal procedures where these are appropriate in order to minimise potential discomfort. Similarly, when investigating the potential value of a new treatment for disease, it is often necessary to apply this treatment over a prolonged period of many weeks to determine its effectiveness. This is therefore not achievable in terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our procedures have been refined considerably through a number of years of experience. We already seek to employ as minimally invasive procedures as possible to achieve our scientific objectives.
However, we also strive to incorporate new methodologies and refinements if they become available and improve outcomes for the animals.

All surgical procedures require our team to ensure animals receive appropriate post operative care and pain management and we discuss these routinely with the named officers within our animal facility.

In some protocols we do expect animals to eventually develop some clinical signs of disease. In these animals we have an enhanced monitoring programme in place to ensure welfare harm to the animal is minimised as much as is practicably possible.

Our experimental animals become habituated to the investigators presence and in some experiments this allows us to train them to come to the side of the pen for food and completion of some aspects of our studies where remote monitoring is a feature. This means that we do not always need to restrain animals to achieve the experimental data that is being sought.

Animals are housed in social groups apart from the immediate post-operative recovery period (24 - 48 hours) when they are individually housed but in pens adjacent to and in sight of their original cohort so as to minimise any potential for separation effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are no specific guidelines covering the animal models that we will employ. However, there are general guidelines covering aspects of our experiments such as blood sampling volumes; these are used to inform our standard operating procedures.

In addition, we regularly receive updates and guidelines through our local Animal Welfare and Ethical Review Board and we monitor websites and ensuing publications that promote best practice and refinements in methodologies including nc3rs.org.uk, felasa.eu, lasa.co.uk.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our animal care facility, NC3Rs manager and ethical review board is responsible for delivering regular updates on 3Rs developments to users. We also monitor outputs published in the scientific literature and appropriate learned society websites.

When a 3Rs advancement becomes available we will consider if these can be adopted into our standard procedures as appropriate; for example, tube handling of mice, and employ them in our course of work as a matter of routine.

The project licence holder and associated investigators also attend a number of committee and scientific meetings each year where animal welfare and experimental practices are a key discussion topic. The members of the research team also attend a number of scientific conferences where animal welfare and refinements are frequent subject matter.

85. Molecular factors affecting sperm production

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (iii) Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

spermatogenesis, genetics, sex ratio, fertility, infertility

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how sperm cells differ from each other, what influences their fertilising capacity and survivability, and how this influences their ability to transmit their genetic information to the next generation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When sperm function is perturbed, in particular how they handle and repair DNA damage, it can lead to male sterility and/or increase the chance of birth defects in the next generation. Studying this will help us develop new fertility treatments, and, conversely, novel methods of contraception. Rarely, alterations in sperm function affect the mathematics of genetic inheritance, meaning that genes may be inherited more than expected by random chance - a concrete example of this being a mouse strain that

produces 60% female offspring and only 40% males. Understanding this "transmission ratio skewing" may allow us to develop ways of regulating the offspring sex ratio in farm animals without needing to cull unwanted animals of either sex (as is the current practice).

What outputs do you think you will see at the end of this project?

The key outputs will be adding to the knowledge of reproductive function in males, and how reproductive function impacts genome evolution. This is expected to lead to journal publications, public engagement and external scientific meetings. As part of this work, we will develop two new mouse lines that will be shared with the scientific community and will permit more powerful and less wasteful studies of male fertility and of DNA damage repair mechanisms.

Who or what will benefit from these outputs, and how?

The basic scientific questions addressed by our work cover fundamental areas of reproduction, and the processes that control sperm production and quality control. This will be valuable to the scientific community in multiple areas of research into fertility. In the longer term we hope this research will contribute towards development of novel methods of sex selection in the livestock industry. An estimated 100,000 male dairy calves and 30-40 million male layer chicks are culled per year in the UK alone. Male pigs are routinely either slaughtered before puberty (leading to economic wastage) or castrated (an animal welfare issue) due to “boar taint”. All of this would be unnecessary if alternative more humane methods of offspring sex selection can be developed as a result of our work.

We will also develop two novel mouse lines that will be of use to scientists studying fertility and DNA damage responses, and share these with the scientific community.

How will you look to maximise the outputs of this work?

We will aim to publish all data that is generated by this work, positive or negative. In addition, we will form collaborations, when natural, to support and increase the probability of generating outputs for publication and public engagement. Novel mouse lines will be deposited in appropriate repositories and shared with the scientific community.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice because the genes we are looking at, and the cellular processes involved, are better understood in mice than in other experimental species. The project does not require any harmful procedures other than breeding. The mice are unlikely to suffer any pain or distress as part of these experiments, as the only predicted effect of the genetic alterations we are studying is an effect on the shape and fertilising ability of sperm. It is necessary to use adult animals as juvenile animals do not produce sperm.

Typically, what will be done to an animal used in your project?

The vast majority of animals used (up to 2500 in total) will not be subject to any procedures other than breeding and post mortem study of tissues following humane euthanasia. The breeding is a regulated procedure because the animals in question are genetically modified, however the genetic modifications we are studying are not expected to produce any effect other than painless alterations defects in sperm shape and function.

If egg collection is required to study the process of fertilisation, this will involve hormone treatment of the females (up to 500) to increase egg production prior to collection of the egg cells post mortem. This requires two intraperitoneal injections spaced a few days apart.

What are the expected impacts and/or adverse effects for the animals during your project?

There are no expected adverse effects other than transient pain following 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)?

The severity is expected to be sub-threshold for the majority of animals (~80%) and mild severity for any that have had injections.

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?
Studies in vitro alone are insufficient to show a candidate cellular abnormality is relevant to a given disease. This requires interpretation of molecular and cellular mechanisms within physiological situations that can only be observed in intact whole organs and indeed whole organisms. In particular, there is no way to make sperm or eggs in cell culture and so live animal work is required to investigate sperm function. Moreover, a key aspect of this project is analysis of transmission ratio distortion - i.e. the rate at which specific genetic changes are passed from one generation to the next. This necessarily involves use and breeding of live animals.

Which non-animal alternatives did you consider for use in this project?

None - there is no alternative to the use of animals. A substantial amount of related work is being done in parallel including analysis of genomic sequence data and previously-published datasets, and cell line work to look at DNA damage in cell types other than germ

cells (which cannot be cultured).
Wherever possible we will use cells derived from our mice rather than the mice themselves to study processes including fertilisation and DNA damage repair, thus sparing mice any unnecessary discomfort.

Why were they not suitable?

Many of the experiments to be done are technically in vitro cell culture experiments using eggs and sperm - however there is no way to produce either eggs or sperm in cell culture, and thus the use of live animals is unavoidable.

For the work focusing on the DNA damage response, while many cell types can indeed be investigated in a cell culture system, other cell types such as maturing sperm cells cannot be cultured and thus once again the use of live animals is unavoidable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Most of our mice will be used for breeding and collection of tissues post mortem. Broadly speaking there are three types of experiment we will carry out.

The first involves studying tissues from GM animals (transgenic or natural mutant) with a known phenotype, along with normal control males, in order to study aspects of sperm development. For this, our estimates of the required number are based on the breeding characteristics of these lines and the number of animals required in order to provide an appropriate level of replication of our results. Typical experiments will use 6-10 individual animals for each genotype tested. Where possible we will archive tissue samples and use these for followup experiments rather than breeding more animals.
The second involves studying tissues from newly-developed GM animals, along with normal control animals, in order to confirm that the modifications have produced the desired effect. Estimates of animal numbers here are derived similarly to the studies on pre-existing GM lines, but will require a more staged approach, with pilot studies on small numbers of animals followed by larger studies to confirm and refine our observations.

The third involves studying sperm, eggs and embryos from GM animals, along with normal control animals, in order to study fertilisation mechanisms and outcomes. In general these will be performed by using hormonal stimulation to induce females to ovulate, followed by post mortem collection of eggs and sperm from animals of interest and in vitro fertilisation experiments. Here, the number of animals used is largely determined by the number of females required to produce enough eggs for the experiments. Our calculations are based on the average number of eggs produced per female, and where possible we will select the strain of mice used to produce the largest number of eggs from the smallest number of females. Typically, in order to detect a ~10% alteration in transmission ratio, 500 eggs are required in each of the experimental and control groups, while a female produces around 50 eggs following stimulation.

Overall, we anticipate using up to 2,000 animals over 5 years in the first two types of experiment, and up to 500 females over 5 years to produce eggs for the third type of experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Variability will be regulated by using littermate controls where appropriate and using stable well- characterised mouse lines such as C57BL/6 where possible. We used the NC3Rs Experimental Design Assistant in designing the planned experiments, and will continue to do so for future grant applications to support 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?

To determine the number of animals required for single-cell analysis of testis function, we will carry out pilot analyses of published datasets in order to determine the minimum required number of biological replicated.

We will maintain all our lines on the same genetic background where possible, to enable sharing of tissues from control animals between experiments. For example, for most of our studies only the male mice are useful (because we are studying testes). Where possible we will make use of the females also, for example as "sentinel" animals to monitory colony health, or as a source of oocytes for experiments involving other mouse lines.

For the line used to study DNA damage, once this has been generated, we will be able to use it to make stable cell lines covering a wide range of different tissues, which we will share with the scientific community.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the premier experimental model for examining gene function in mammals, being permissive for transgenic manipulation, rapidly developing, and extremely well genetically characterised. Hence, we propose the use of mice as data collected from our studies can be readily compared with that already published and, in parts, serve as control data when comparing with transgenic models.

Why can’t you use animals that are less sentient?

Alternative laboratory species that are less sentient include fruit fly, zebra fish and nematode worm (c elegans). However, in all of these the organisation of the testis differs

very substantially from the mammalian testis, and so they are not appropriate biological systems to investigate mammalian sperm production and fertilisation. Juvenile animals cannot be used as they are reproductively immature and do not produce sperm/eggs. Since we hope to ultimately develop sex selection protocols for farm animals, it is important to work in a mammalian system to ensure our data is likely to translate well into these species in due course.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At our establishment we have already moved to tube handling for all animals, which reduces fear and distress. The only procedure liable to cause any welfare impact on the animals is injection, which will be performed by appropriately trained and supervised personnel. Animals will be closely monitored during all procedures and where possible we will consider refining existing techniques or incorporating new methods to minimise any suffering to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Ullman-Cullere et al 1999 'A Rapid and Accurate Method for Assessing Health Status in Mice' Lab Animal Science; 49(3):319-323.

Wilkinson et al 2019 'Progressing the care, husbandry and management of ageing mice used in scientific studies' Laboratory Animals 0(0):1-14.

Turner et al 2011 'Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider' J Am Assoc for Laboratory Animal Sci 50;600-613.

Workman et al 2010 'Guidance for the welfare and use of animals in cancer research' Br J Cancer 102;1555-157.

Diehl et al, 2001 'A good practice guide to the administration of substances and removal of blood, including routes and volumes' J. Appl. Toxicol. 21, 15–23.

'The Design of Animal Experiments: Reducing the use of animals in research through better experimental design' (Festing et al 2nd Ed).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3Rs website provides an outstanding resource of information. We will review this website on a regular basis for relevant information. In addition, our local AWERB provides us with an excellent platform to discuss the 3R's, where this is a long standing item on every agenda. I will attend appropriate seminars and conferences covering breeding and transgenesis technology.

Home Office

86. Identifying novel biologic drugs for the treatment of liver disease

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

NASH, Liver fibrosis, Bispecific drug, VNAR, scFv

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 identification of new therapeutic drugs for the treatment of Non-Alcoholic Steatohepatitis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Non-Alcoholic Steatohepatitis (NASH) is a progressive form of non-alcoholic fatty liver disease (NAFLD), which is the most common chronic liver condition in Western populations. NASH is characterised by chronic inflammation and an extensive accumulation of fat in the liver and, although it can affect people of any age, most patients are between the ages of 40 and 60 years. It is a silent chronic disease with few or no symptoms (tiredness, unwellness and discomfort in the upper right side of the abdomen). However, over time, it can develop into more serious conditions, such as liver cirrhosis or liver cancer, where liver transplant is the only option. In addition to liver-related morbidity

and mortality, patients with NASH are at an increased risk of cardiac morbidity and mortality This disease affects 35 M people globally and with patient numbers growing rapidly, the market is expected to rise towards £18 B by 2026. Critically, to date, there is no medication approved by Food and Drug Administration (FDA) and therefore, this innovative work emerges as an opportunity to develop first-in- class (i.e. drugs that use a new and unique mechanism of action) therapeutic candidates for the treatment of this devastating disease.

What outputs do you think you will see at the end of this project?

We are striving to identify new therapeutic drugs for NASH but due to the nature of drug discovery this is a long-term aim. In the shorter term, this would certainly lead to new intellectual property, peer- reviewed publications and conference presentations.

Who or what will benefit from these outputs, and how?

Longer term would be the clinical benefit from these new modalities by NASH patients, resulting in a huge humane and financial benefit. Given the novel approach of combining different targeting moieties there would be invaluable learning regarding the engineering behind this approach such as the stability of the linker, the impact on PK/PD of a bi-functional molecule, the stoichiometry of interaction and criticality that affinity plays in such a product.

How will you look to maximise the outputs of this work?

Primarily to gain more support to progress the work into later stage development as well as new IP, conference communications and scientific publications.

Species and numbers of animals expected to be used

- Mice: Objective 1: 1,000 mice; Objective 2: 1,000 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.

To date, there is no medication approved for the treatment of NASH, the most aggressive form of NAFLD. In this regard, preclinical models are needed to fully understand the pathophysiology of this disease and identify novel therapeutic opportunities. Over the last decades, several animal models of NASH have been described. Among them, induction of fibrosis by intoxication with carbon tetrachloride constitutes one of the most characterised models and a robust tool to study the ability of effective compounds to reverse fibrosis. Application of this hepatotoxin induces acute and chronic liver injury in animals, leading to fibrosis where spontaneous reversion is minimal. Therefore, this model constitutes an excellent tool to study and identify antifibrotic therapies.

We use rodent models because their genetic and biological characteristics closely resemble those of humans, and many symptoms of human conditions can be replicated in

mice and rats. The CCl_4 model can be applied to both rats and mice. However, mice are preferred, because of a higher metabolic rate of CCl_4 compared to rats. Young adult C57BL/6 mice (8-10 weeks old) will be used to perform these experiments. Although BALB/c mice shows more susceptibility to CCl_4 than C57BL/6, higher level of severity and a more specific Th2-type response are obtained by using this strain. C57BL/6 shows intermediate susceptibility to CCl_4 and is the strain most frequently used to study fibrosis in vivo by other groups. Likewise, previous studies from our group were performed with male C57BL/6 mice, and so, this strain will be used to be able to compare our results with those obtained before by our group and others.

Typically, what will be done to an animal used in your project?

For protocol 1:

Liver fibrosis will be induced by bi-weekly intraperitoneally application of CCl_4 for up to 8 weeks (0.8-
1.2 ml/Kg). Control mice will be established by injecting with olive oil vehicle. An SOP will be strictly followed covering CCl_4 preparation, vehicle (olive oil), dose, treatment duration and post-injection monitoring.

24-72 hours after the last dose of CCl_4 or olive oil of the last week of treatment, animals will receive either fluoro-labelled test article or control article. Then, mice will be humanely killed by Schedule 1 method at different time points (0 - 24 hours) and tissue and blood samples will be removed for analysis.

For protocol 2:

Liver fibrosis will be induced by bi-weekly intraperitoneally application of CCl_4 for up to 8 weeks (0.8-
1.2 ml/Kg). An SOP will be strictly followed covering CCl_4 preparation, vehicle, dose, treatment duration and post-injection monitoring.

Between the two doses of CCl_4 of the last week of treatment, animals will receive either DMSO (vehicle for toxin), free toxin, PBS (vehicle for test artcicle), control article, control article conjugated to toxin, test article conjugated to toxin or test article. This test article will be the same used in protocol 1 but, instead of being labelled with fluorescence, it will be conjugated to a toxin. 24-48 hours later, animals will receive another dose of CCl_4. Finally, mice will be humanely killed by Schedule 1 method 24 – 48 hours after the last dose of CCl_4 and tissue and blood samples will be removed for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects post-injection: animals will experience acute abdominal pain after CCl_4 administration (up to 100%). In less than 1% of the animals, the injection could breach the gut provoking peritonitis, which will be identified by rapid appearance of clinical signs of sepsis, such as swollen abdomen, lethargy, starry coat, hunched posture and reduced normal activities. If this happens, the animal will be immediately sacrificed by a Schedule 1 method. Furthermore, male mice could exhibit aggressive behaviour due to injections.

Adverse effects as a result of repeating doses: mice injected with CCl_4 do not usually lose weight, but they might not gain weight with growth like control mice (olive oil vehicle). Body weight will be closely monitored over the course of the experiment (at least twice a week). Those animals that actively loss more than 20% of their starting body weight or show any critical alterations in their behaviour will be humanely sacrificed 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)?

Moderate, only in those animals treated with CCl_4 (50% mice in protocol 1 and 100% mice in protocol 2). Mice injected with vehicle in protocol 1 (50%) will be 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?

At present there is no alternative in vitro or ex vivo models for liver fibrosis. Although cell lines are available, they do not represent a true substitute for the real tissue. Given the driver is to develop a clinical drug candidate, understanding the effect of the test article within the complexity of a living animal is by far the most translatable for late stage drug development.

The team are always actively looking (via NC3Rs, CrackIT, FRAME, Norecopa, Understanding Animal Research, etc) at alternative systems for this and other programs of work, such as 3D tissue culture, spheroid and organoid models and liver chips. However, the whole living body is necessary to address the potential of our biologics in terms of specificity and effectiveness.

In vitro cell-based work will be performed in activated hepatic cells to assess these new domains and select the best one for further in vivo studies. This represents one of the go/no decision points in our protocols and only if significant in vitro cell-based work has been completed, the next step involving animal models will be performed.

Which non-animal alternatives did you consider for use in this project?

All in vivo work will only be conducted after significant in vitro cell based work has been completed to select and fully characterise the final lead product. Thus, binding and functionality of our compound will be previously analysed in vitro by using activated stellate cell cultures.

This represents one of the go/no decision points in our protocols and only if the results show good binding affinity and functionality, the next step involving animal models will be

performed.

Organoids and liver chips will be considered future projects to analyse dose-response toxicity of our compound in humans.

Why were they not suitable?

Although using activated cell lines is a good early step to analyse the functionality of the bispecific compound, they do not represent a true substitute for the real tissue. Given the driver is to develop a clinical drug candidate, understanding the effect of the test article within the complexity of a living animal is by far the most translatable for late stage drug 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?

Based on previous work conducted and significant statistical data analysed, sample size calculations have been carried out to determine group sizes for both objectives. Furthermore, an extra number of animals has been considered to include some margins in case animals become ill and to perform pilot studies. For Objective 1, 5-6 animals will be used for each group. Objective 1 involves 4 different experimental groups with 5 different endpoints each and therefore, a total number of 100-120 animals are needed for each test article. For Objective 2, an effective sample size of 8-9 is required for each group. Objective 2 includes 7 different groups, and therefore, a total number of 56-63 animals are needed for each toxin-conjugated test article.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Sample size calculations using different tools, such as the one suggested by the national statistical advisory group, G*Power 3.1.9.7, have been performed to determine group sizes for both objectives.

Since female animals show less consistent response to CCl_4 treatment, only male C57BL/6 mice will be used to reduce the number of animals per experiment.

Finally, intraperitoneally injection demonstrates less mortality than inhalational CCl_4 or gavage application and so, a smaller number of animals is needed 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?

Pilot studies will be used to select the right dose and length of CCl_4 treatment and test articles in order to and avoid mortality, which would increase the number of animals

needed. In addition to that, pilot studies will be carried out for fluoro-labelled and toxin-conjugated test articles to identify dosage with no adverse effects.

Blood and multiple tissues from each individual mouse will be collected at the end of the study. Multiple analysis will be performed from each animal, thereby obtaining maximum 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.

Experimental liver fibrosis can be induced in mice by genetic manipulation, change of diet, surgical intervention (e.g. bile duct ligation, BDL) and application of hepatotoxins.

Genetic manipulations rarely develop liver fibrosis as such and need a second stimulus for disease induction. Moreover, using genetically modified models would require a high number of animals, and therefore, it is not considered for this project licence (application of the principle of 3Rs, Reduction). Diet-based models are excluded because they do not reproduce human NAFLD progression into NASH (Yanguas et at, 2016. Arch Toxicol. 90(5): 1025–1048).

Our single domains have been selected against proteins that are expressed on activated hepatic stellate cells (aHSCs). In this regard, there is a study that demonstrates how hepatotoxic (CCl_4 models in particular) and cholestatic (BDL) liver injuries activate different subsets of fibrogenic myofibroblasts: CCl_4 administration activates preferentially stellate cells, whereas BDL originates activated portal fibroblasts (Iwaisakoa et al., 2014. PNAS. 111(32), E3297–E3305). Additionally, CCl_4 is the toxic model of liver fibrosis most commonly used worldwide and has been applied in more than 50,000 studies so far. It is the best characterized in terms of histological, biochemical, cell and molecular changes associated with the development of fibrosis and has been established as a standard method for the assessment of anti-fibrotic therapeutic agents. Compared to the other models, CCl_4-induced liver fibrosis is easy to set up, highly reproducible and reflects the mechanisms involved in human liver fibrosis (Janakat and Al-Merie, 2002. J Pharmacol and Tox Methods.48, 41-44; Scholten et al, 2015.
Laboratory Animals. 49, 4-11; Yanguas et at, 2016. Arch Toxicol. 90(5): 1025–1048, Hansen et al, 2017. Drug Discov Today. 2017; 22(11):1707-1718). As we study non-alcoholic steatohepatitis, ethanol will not be considered for this project licence.

Intraperitoneally injection shows less development of ascites than prolonged inhalative CCl_4 treatment, which promotes severe cirrhotic changes in the liver (Scholten, D et al. 2015. Laboratory Animals 49 (S1) 4-11; Domenicali, M et al. 2009. J Hepatol. Dec;51(6):991-9). Application of CCl_4 by gavage will not be used as it leads to significant distress and high rates of early mortality (Scholten, D et al. 2015.

Laboratory Animals 49 (S1) 4-11), which would require higher sample numbers (application of the 3R's principle: Reduction and Refinement). Also, intraperitoneally administration of CCl_4 was the route used in our original research and has been widely described as the most chosen application in the literature for C57BL/6 mice because it causes less pain, suffering, distress or lasting harm to animals than inhalational CCl_4 or gavage. A 27G short needle will be used, changing needles between different animals. Pilot studies will be carried out to address the correct dose and length of CCl_4 treatment, and all the procedures will be performed following strictly both Laboratory Animal Science Associaton (Lasa) good practice guidelines and a SOP. Animals will be closely monitored in terms of body weight loss or abnormal behaviour and humane end points will be applied all along the protocol.

Why can't you use animals that are less sentient?

Although CCl_4 accelerates and exacerbates liver fibrosis in mice compared to other methods, such as genetic or dietary models, a minimum of 4 weeks is needed to develop liver fibrosis, and therefore, young adult mice (8-10 weeks old) are required to perform this procedure.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be handled for at least a week prior to any experiment, which will result in animals being relaxed and calm at the time of injection, and therefore, anaesthesia will not be needed. A previously drawn-up chart of weight/dosages could be used to inject the animal with little or no delay after it is weighed, thus minimising stress and unnecessary handling. The person injecting the mice will be very experienced in the intraperitoneal administration technique. In addition, pilot studies to determine minimal dosage and length of treatment required for scientific outcome will be performed and will help to minimise occasional mortality. Half of the dose will be used in the first injection to allow animals to get used to the treatment. Likewise, if a slight weight loss (0.5 g) is observed during the treatment 3 times in a row (i.e. 1.5 g of weight loss between 3 injection times), only half of the dose will be applied in the following injection.

To minimise pain or distress to animals, analgesia (e.g. buprenorphine) will be administered into mice 30-45 minutes before intraperitoneal injection. Animals will be closely monitored to identify any pain associated behaviour, such as abnormal posture (hunched), piloerection, social isolation or abdominal distention. In addition to the pain relief, other measures might be considered if necessary. These would be discussed with the NACWO and NVS. If any of these signs is critical, animals will be humanely sacrificed by a Schedule 1 method. In addition, body weight will be measured at least twice a week and any mouse showing weight loss greater than 20% will be humanely sacrificed by a Schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance on the Operation of the Animals (Scientific Procedures) Act 1986

(https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/662 364/Guidance_on_the_Operation_of_ASPA.pdf)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

National Centre for the Replacement Refinement & Reduction of Animals in Research website (https://www.nc3rs.org.uk/) will be periodically reviewed to be informed about any advances in the 3Rs (Replacement, Reduction and Refinement) and implement them effectively during the project.

87. Oncology Models

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Cell therapy, T cells, Adoptive cell transfer, 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?

The overall aim is to support the research and development of new medicines to treat 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?

Cancer is the second leading cause of death globally with estimated death rates of around 10 million per year (World Health Organisation 2021), along with estimates suggesting a 1 in 2 chance of the population getting cancer in their lifetime. It is clear there remains a high unmet medical need for successful treatments of cancer. Environmental and genetic factors also play a part in the ever-evolving battle against cancer and, despite continuous efforts worldwide over the years, new and more effective medicines are still very much in demand.

What outputs do you think you will see at the end of this project?

Validation of new therapies, and existing therapies where effectiveness has already been confirmed, with potential successful advancement into clinical trials.

Modification and enhancements of therapies currently in clinical trials. Successful modified therapies may be advanced to clinical trials to demonstrate improved long-term effectiveness.

Generation of new information on the mechanisms of therapy effectiveness, with identification and evaluation of potential safety concerns.

Who or what will benefit from these outputs, and how?

People benefiting from the new therapies would include severely and terminally ill cancer patients who may have exhausted other treatment options. These new therapies would aim to give a complete anti- tumour response or an increase in the patient's survival. There is potential that within the 5 years of this Project Licence we can achieve our key outputs.

Shorter term benefits from the work carried out on this Project Licence will include a gain of scientific knowledge as the therapies progress through the drug discovery process, including the design of clinical trials. This will allow critical decision making and prioritisation of medicines.

How will you look to maximise the outputs of this work?

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: 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.

Adult mice, usually with a compromised immune system, will be routinely used on this licence. These mouse strains are often lacking in many aspects of a normal immune system, such as T, B and Natural Killer (NK) cells. This results in the mice accepting tumour cell engraftment and therapies, where immune competent animals would reject the cells and therapies as foreign bodies.

Typically, what will be done to an animal used in your project?

It is expected that most animals will have tumour cells administered to them. As these tumours grow, they will be closely monitored by either manual volumetric measurements (e.g. using callipers), imaging systems such as MRI, or a combination of both. Animals that

have imaging procedures will be under general anaesthesia and may be injected with substances, such as contrast agents, to show tumour formation and characteristics, such as blood flow. Animals may be treated with test substances, cells and therapies, usually by injection, to ascertain their effectiveness and persistence at aiding the reduction and elimination of the tumours. Conscious animals may have periodic blood samples taken from the tail veins. Larger blood samples may be taken at the end of the studies whilst under general anaesthesia from major blood vessels or the heart. These animals will never regain consciousness.

Studies will generally be 30-40 days in length but will be dictated by factors such as the rate of tumour growth and the therapies effectiveness.

What are the expected impacts and/or adverse effects for the animals during your project?

The primary expected impact on the mice is tumour formation, which could last the duration of any study. Depending on the location of the tumour, the impacts on the normal behaviour, pain and wellbeing of the mice are likely to be minimal. For instance, it is unlikely that tumours implanted subcutaneously (under the skin) would have a significant adverse effect on the mouse, unless ulceration of the skin at the tumour site is seen. The effects on mice implanted with tumour cells intravenously may be greater due to the multiple locations where tumours may form. Weight loss of the mice is not usually seen, though tumour burden can impact this. For tumours that form within multiple organs, close monitoring of these animals along with imaging techniques will allow us to humanely euthanase animal before significant impacts on the animal's welfare are seen.

Immune mediated diseases such as Graft vs Host Disease (GvHD) may be seen in animals, usually occurring 25 or more days after receiving a cell therapy. This can cause several adverse effects, including weight loss, lethargy and limb swelling, but careful monitoring of this will ensure animals showing these signs are humanely euthanased before impacting the animal's 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)?

Approximately, 70% of the mice used on this licence are likely to experience moderate severity. The remaining 30% would be expected to experience mild severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are required to be used on this project to mimic the complex environment and interactions within the human body and the interactions that occur with cancers. These interactions play key roles in developing new therapies (medicines). Features, such as the structure of the tumour and blood flow cannot be achieved outside of an animal.

Assessment of compounds can be carried out in vitro (without using animals), however, it is primarily the interactions of the potential therapeutics with the complex in vivo environment which allow us to determine a more accurate profile of effectiveness. Assessments without the use of animals will be used prior to using animals. These in vitro experiments may look to triage therapeutics by combining tumour cells and potential therapies. The therapies most likely to benefit patients will be chosen from these experiments and may then progress for further testing in mice, where additional aspects that cannot be created in vitro, such as the effect of the therapy on the rest of the body, can be assessed.

Which non-animal alternatives did you consider for use in this project?

Many in vitro experiments already incorporated into the study designs are suitable at determining tumour cell characteristics, therapy profile and the effect of combining these to show the killing effect of the therapy on the cancer cells.

As well as these in vitro experiments, there are a number of other alternatives that have been considered, such as 'tumour-on-chip' technology. These devices aim to recreate relevant features of the tumour physiology without the use of animals.

Organoid technologies have also been researched and some evaluated in house and in collaborations. These innovative approaches aim to recapitulate the tumour microenvironment and to demonstrate interactions between the tumour cells and therapies.

Other complex in vitro models evaluated in house include ‘air liquid interface’ and ‘epi-intestinal models’, which look to model the in vivo gut environment amongst other things.

Trujillo-de Santiago G, Flores-Garza BG, Tavares-Negrete JA, et al. The Tumour-on-Chip: Recent Advances in the Development of Microfluidic Systems to Recapitulate the Physiology of Solid Tumors. Materials (Basel). 2019;12(18):2945. Published 2019 Sep 11. doi:10.3390/ma12182945

Why were they not suitable?

The in vitro experiments already performed by the company as well as other non-animal alternatives researched, such as organoid research and ‘tumour on a chip’, do not fully replicate the complex interactions between the body and tumour, but also do not demonstrate the time course and patterns of tumour growth, metastasis and establishment of a blood supply and interactions with the immune system. Cell therapies also have complex relationships within the body as they migrate and replicate. These interactions cannot be replicated or fully understood without the use of animals.

Other in vitro models researched in-house and in external collaborations, such as ‘air liquid interface’ and ‘epi-intestinal models’, produced unreliable data and tissue quality deterioration over time.

Organoid and ‘Tumour on a chip’ technology has potential and continues to be developed, but at present creates complex technical difficulties for the scientist and longer experiments can cause issues with maintaining healthy samples. As these technologies continue to be developed, further consideration to use them will be made, but at present there are no suitable alternative to completely replace using animals to achieve the objectives on this licence.

Sontheimer-Phelps, A., Hassell, B.A. & Ingber, D.E. Modelling cancer in microfluidic human organs-on- chips. Nat Rev Cancer 19, 65–81 (2019). https://doi.org/10.1038/s41568-018-0104-6

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimated animal numbers have been calculated from the experience gained from previous Oncology based Project Licences and projections of 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?

The company has a ‘Robust Study Design’ initiative in place to ensure the appropriate number of animals are used on each study and that data collected is done so in a scientifically justified way. As part of this initiative, a statistician will be consulted to advise on all aspects of experimental designs to ensure the most suitable number of animals are used on each experiment. The aim would always be to reduce the number of animals used where possible, whilst not compromising on the objectives and quality of the results obtained. An example of this would be ensuring there are enough animals in each dose group to show statistical differences, though having no more than actually required to demonstrate this.

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 will be scientifically reviewed by an internal expert panel prior to commencing, which ensures a broad range of experienced individuals will comment on the overall study design and recommend any improvements. This includes the overall aims of the experiment and optimal sharing data to reduce replication and overall animal usage.

On occasion, small pilot studies may be undertaken to confirm readouts, such as successful tumour growth, if no suitable previous work or publications can demonstrate this. This may then lead to further optimisations, such as cell concentrations, in larger growth kinetic studies.

To reduce variation in biological readouts we will ensure that our facilities provide a constant optimal environment suitable for the strain and species, and we will control the

number of personnel involved in making any subjective experimental measures e.g. tumour measurements by callipers. By minimising variables, we aim to ensure that animal group sizes are the smallest possible to achieve the scientific objectives of the study.

Due to the complexity of the projects on this licence, it is routine that many organs and tissues are investigated by different groups within the company, including histopathology, therapy levels within the target site and imaging. Careful planning of this prior to studies starting allows for the animals to be used in the most efficient ways possible and reducing overall numbers 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 species used on this licence will be mice, most of which will have deficient immune systems to allow human cancers to grow. The most appropriate strains will be selected for each animal study, which exhibit the desired traits that allow for successful tumour growth. This gives a greater chance of the treatment working in a clinical setting. These mice will be maintained and handled in such ways to maintain a barriered environment so not to compromise the health of the animals, including sterile caging and bedding. They will be handled by cupping or tube handling whenever possible. Some of the mice used may have some phenotypic traits that require careful monitoring, therefore detailed scoring sheets will allow for these observations to be captured in a clear and consistent manner.

The volumes of injections 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. Animals will be warmed prior to blood sampling to allow for successful blood collection. This will be kept to the shortest time possible with an aim to give the smallest impact to the animal.

Tumour cells will be implanted into the mice and subsequent tumour growth routinely monitored throughout the course of the study, either by volumetric measurements (e.g. manual callipers), imaging techniques under anaesthesia, or a combination of both. There may also be occasions where technological advances allow for automated tumour measurements to be carried out on conscious animals. Bodyweight will be recorded at regular intervals which can give an indication of the effects of tumour burden on the animals. Maximum tumour volume thresholds for individual studies will be set to the minimum size to complete the study objectives instead of aiming for the maximum allowed on this licence. This aims to minimise the overall burden on the animal where possible.

Animals will be routinely group housed with appropriate caging substrate, nesting material and environmental enrichment. As animal numbers decrease during the study (e.g. as tumour volumes reach the set points and animals are euthanased), then there may be

occasion when the remaining animals within that cage are singly housed.

Why can't you use animals that are less sentient?

Animals with complex bodily interactions along with interactions with cancers are required to mimic cancers in human patients. Routinely, the adult mice used on this licence have compromised immune systems that have been specially developed for this type of research, allowing for human cancers to grow within their body. The therapies being developed also have complex interactions within the body and these need to be researched prior to progressing into patients. Studies of cancer in mice are extremely useful for understanding the cancer biology and provide insights into the tumour growth, progression and metastasis.

Although research into cancer models in less sentient animals, such as zebra fish, is taking place within the oncology community, there is no less sentient animals available that can provide as complete package of scientific data that mice can provide.

Due to the length of studies on this licence, it is not possible to use terminally anaesthetised animals for the duration of the experiment, however animals will be placed under general anaesthesia for procedures, such as tumour implantation, imaging techniques and terminal sampling, to reduce the stress to the animal.

Zhang W, Moore L, Ji P. Mouse models for cancer research. Chin J Cancer. 2011;30(3):149-152. doi:10.5732/cjc.011.10047

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals on studies will be monitored once daily as a minimum, and any adverse clinical signs that could be treatment related will be recorded and, where beneficial, scored by reference to a specific scoring system for each type of experiment, therefore allowing monitoring of potential trends and modification of treatments if necessary.

Animals will be tube and cup handled when possible to reduce stress, though scruffing of the mice will be required to manually measure tumours. Modified and newly developed restrainers will be used when possible for blood sampling and dosing inside barriered cabinets, allowing easier operation for the staff and with an aim to decrease the stress caused to the mice. These restrainers aim to confine the mice less during the procedure, allowing for greater airflow and allowing uninhibited visual monitoring of the animals throughout.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Aspects of the published principles and philosophies behind the PREPARE (2018) and original ARRIVE (2010) guidelines have been and continue to be incorporated into the sponsoring company's internal project planning, standards of care and standard operating procedures. All work carried out under authority of this licence will undergo assessment of the study design during planning stages as part of a peer review process that is based on those guidelines and will include statistical consultation. Facilities and processes are audited by independent bodies such as AAALAC International.

The company uses the following as a guide for setting maximum blood sampling and

dosing limits:

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.

The following publications will also be used as guidance:

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

Ullman-Culleré MH, Foltz CJ. Body condition scoring: a rapid and accurate method for assessing health status in mice. Lab Anim Sci. 1999 Jun;49(3):319-23. PMID: 10403450

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 raises this type of information and discussion points at the institutional Animal Welfare and Ethical Review Body (AWERB). The licence holder for this work attends both these groups and is also separately aware of the 3R's related work of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the Royal Society for the Prevention of Cruelty to Animals (RSPCA). Experience to date has shown that 3Rs issues and advances are highlighted, discussed and actions are implemented within the company centrally and effectively via these forums.

88. Neural Mechanisms of Social Behaviour

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cognition, neural circuits, neurons, development, social behavior

Animal types	Life stages
Rats	embryo, neonate, juvenile, adult, pregnant
Mice	embryo, neonate, juvenile, adult, pregnant
Gerbils	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the brain mechanisms involved in social perception, how these mechanisms are altered in development and eventually in disease. We also aim to explore evolutionary comparative aspects of the examined 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?

Neurodevelopmental and mental health disorders are associated with alterations in social sensory processing, development and cognition. The primary aim of our research is to understand how the neural circuits of a healthy brain support social behavior, cognition, development and sensory processing. The result of these findings will support a basis on which to build an understanding of how the brain is altered in disease states and how to remedy these dysfunctions with treatment. Further unique aims of this work are to examine

neural activity and the underlying mechanisms which are most naturally relevant to the animal as well as evolutionary comparative aspects of the examined processes.

What outputs do you think you will see at the end of this project?

The result of this work will lead to a greater fundamental understanding of how the brain supports social behaviour, attachment, cognition, development and sensory processing. These fundamental discoveries of how neurons function in these processes will provide a basis for understanding how dysfunction occurs in disease and mental health disorders.

Who or what will benefit from these outputs, and how?

Our work will build a foundation of knowledge regarding the basis of neural function and how these processes may have evolved. We expect the impact of our work to have a significant impact on basic research. Once we build a fundamental understanding of brain function in social cognition, further studies will pursue an understanding of how these neural processes are affected in disease. A further, more long-term effect, will be the development of treatments to reverse the dysfunctions in disease and mental health disorders.

How will you look to maximise the outputs of this work?

We will aim to publish datasets of all of our studies regardless of outcomes. We will also maximize outcomes of our research through collaborations as well as presentations in international conferences.

Species and numbers of animals expected to be used

- Rats: 3000
- Mice: 30
- Gerbils: 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.

We will use rodents for this research, which show robust social behaviours and are ideally suited for studying the neural circuits underlying these behaviours. We plan to assess how regions of the brain and genetically defined pathways support social behaviour. We will ask questions related to the structure and function underlying development of social attachments, social bonding and recognition in the context of naturally relevant social behaviours. We will assess rodents at all developmental stages including prenatal stages of development until adulthood.

Typically, what will be done to an animal used in your project?

Animals used in this project will undergo surgery, injections of substances, cranial implants, culling under anaesthesia and behavioural assessments. As social behaviours are highly dependent on identity, animals will also be identified with tattooing as well as

radio-frequency identification microchipping to ensure that identification can be reliably performed without fail. Experimental durations will range from acute (1 day) to several weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential adverse effects include possible death under anesthesia in survival surgeries, pain following surgical procedures which will be significantly reduced with analgesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severities are considered to be moderate and mild for all procedures. For survival surgical procedures in juvenile animals, death from anaesthesia in and following surgery may occur more frequently. Death rates occurring under anaesthesia in juvenile animals will be monitored and reported.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to understand neural circuit function in complex behaviour, an approach using multiple technically advanced methods are required. These advanced methods include imaging, electrophysiological, transgenic and pharmacological manipulations of the mammalian brain. Experimental systems that allow this are limited and must involve the use of vertebrate animals. We will utilize in vitro preparations where possible to assess reduced aspects of cellular function, however these approaches are limited and do not faithfully reflect the properties of an intact circuit.

Which non-animal alternatives did you consider for use in this project?

Human and ex vivo tissue investigations.

Why were they not suitable?

Investigations of the neural circuits underlying social behaviour in a non-intact animal is not possible as these behaviours can only be performed in interacting animals. Observations of social behaviour in humans is limited in scope as there are not possibilities to manipulate elements of the neural circuits in a highly resolved manner as we are able to in the rodent.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate the given numbers of experimental animals based on usage statistics of previous publications as well as information provided from other license holders at our establishment. The numbers required for upkeep and desired out-breeding of lines are derived from estimates provided by the animal facility. To stay updated on optimized experimental design, we will regularly seek guidance from biostatistical experts employed by our establishments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the experimental design stage of the project, we have made plans to perform pilot studies where possible before planning for large cohorts. All experiments will be conducted according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies whenever possible to assess the potential for publishable results. We will additionally perform computational modelling and analysis of datasets to maximize the information extracted from our datasets.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rodents as an animal model and will aim to assess neural activity and the outcomes of circuit and pharmacological effects on behaviour. We aim to understand neural function and circuits in the healthy brain which will provide a foundation for understand brain dysfunction in disease. Thus, this approach will reduce the number of animals subject to adverse states in severe disease models.

We will utilize wild-type and transgenic rodents without harmful phenotypes as animal models meaning that rodents in the unperturbed state will experience no harmful effects.

We have identified species- specific behaviours in terms of kinship and mate choice. For this reason, we aim to use an evolutionarily comparative approach to uncover differences in neural circuit structure and function which contribute to these species-specific social behaviours. We will utilise rats, gerbils and mice for their species specific social behaviours. In contrast to rats and mice, gerbils display characteristic pair bonding, parental and inbreeding avoidant behaviours which are significant to our research goals.

Why can’t you use animals that are less sentient?

As the goal of our research is to understand complex cognitive processes involved in social behavior, it is not possible to examine this in less sentient species.

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).

89. The hematopoietic system in normal development, ageing and cancer.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Blood cell production, Hematopoietic stem cells, Leukemia, Breast cancer, Ageing

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand how blood cells are normally produced, and how this process is perturbed in ageing and haematological disorders and cancers. The ultimate goal is to identify molecular targets critical to disease developmentand use these therapeutically.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 critical for oxygen transport, vascular integrity and immunity, and deterioration of blood cell production with age contributes to frailty and loss of adaptive

immunity. In addition, the blood system can both give rise to cancers and contribute to tumour progression in other tissues.
Understanding how normal blood cell production is regulated, and identifying the mechanism by which the production and function of blood cells is altered in pathological conditions, is therefore important for the development of regenerative and therapeutic strategies that can maintain population health and treat malignant disease.

What outputs do you think you will see at the end of this project?

The blood system is important for the body to function and for its ability to fight off infections. The normal production of blood cells is disrupted during ageing, contributing to frailty and susceptibility to infections, and in blood cancers, ultimately leading to loss of normal blood production and death. In order to better understand how to counteract the detrimental changes to blood cell production during ageing and in blood cancer we will here perform genetic experiments to improve our knowledge of normal blood cell production, and use this knowledge to better understand how to counteract the changes that occur to the blood system in aged individuals and pathological states.

Specific outputs will be:

An improved understanding of the nature and function of blood forming stem- and progenitor cells that will allow us to understand how blood cell production normally works and to better diagnose and understand disorders of the blood system.

An improved understanding of the response of the blood system to stress, including inflammation and bleeding.

Genetic tools for the identification and genetic manipulation of blood forming stem- and progenitor cells.

Generation of accurate pre-clinical models for the study of hematological disorders that can be used for drug development and testing.

Therapeutic strategies to counteract the loss of red cell and lymphocyte production during normal physiological ageing

Therapeutic strategies to treat blood cancer, particular those affecting the myeloid cell-producing stem- and progenitor cell compartment

Who or what will benefit from these outputs, and how?

The fundamental knowledge generated about the blood system will be of use to the scientific community, as will the improved tools for the study of blood cell production that will be generated. These outputs will provide benefits within the time frame of the project.

In the longer term (5-10 years) clinicians, patients and the general public will benefit from improved therapeutic strategies against age-related anaemia and declining immune function, as well as better diagnosis and/or treatment of blood cancers.
How will you look to maximise the outputs of this work?

We will disseminate our results at scientific conferences and in publications, as well as through public engagement and engagement with patient groups.

We will make any new genetic tools and disease models available to the scientific community through collaboration and through 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: 42500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 the mouse as species: In this project we will use genetic mouse models to study the normal function of the hematopoietic system, how ageing affects its function, and how blood cancers develop. We use mice because they represent the species with a hematopoietic system that most closely resembles the human hematopoietic system where genetic modification is accurate and accessible, and where inbred strains exist that can be used for transplantation while avoiding immune rejection issues. Therefore, studying normal and perturbed hematopoiesis in the mouse provides information that is highly relevant to human disease and ageing. In addition, the mouse is sufficiently similar to humans to allow human cells to be propagated in vivo using xenografting of immune-deficient animals.

The choice of life stages: The hematopoietic system reaches its adult state only after birth, around week 3-4. As we wish to study the baseline and stress hematopoiesis and how hematopoiesis changes with age we will use juvenile or adult mice in non-breeding protocols, as well as aged mice in those protocols where ageing is studied. We will primarily study myeloid-lineage hematological malignancies, which are common in adults, but not children, and are closely associated with ageing

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.

Experimental mice will undergo the following typical experiences:

Induction of gene expression by substance administration (tamoxifen, 3 times by oral gavage), followed by blood sampling (8 times over 4 weeks) and exanguination under terminal anaesthesia.

Induction of gene expression by substance administration (tamoxifen, 3 times by oral gavage), leading to the development of a hematological malignancy over 12 months. A single bone marrow sample will be taken. Mice will then receive daily substance administration for 2 weeks by oral gavage or intraperitoneal injection. Blood samples will

be taken 12 times over a 4-week period. Mice will be killed by schedule 1 method.

Lethal irradiation and bone marrow transplant, followed by 4 weeks recovery. 3 times oral gavage with tamoxifen at 2-day intervals. Blood samples will be taken 8 times over a 4-month period. Mice will be killed by a schedule 1 method.

Minor surgery under general anaesthesia, substance administration (daily for 4 weeks), sublethal irradiation and bone marrow transplant, followed by 4 weeks recovery. Blood samples will be taken 8 times over a 4-month period. Mice will be killed by a schedule 1 method.

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, and the experiment will be terminated if more than moderate anaemia develops. Irradiation prior to bone marrow transient plantation can cause transient mild anaemia, expected to last 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)?

Mouse:

Mild severity: 65%

Moderate severity: 35%

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 physiological processes that we will study (hematopoiesis, ageing, blood cancer development) are highly complex, as they involve the dynamic interaction of multiple cell types, including systemic, metabolic and environmental changes over time and the stepwise acquisition of genomic and epigenomic changes. There is currently no adequate methodology for modelling these processes in vitro, in particular since the molecular and environmental changes involved are only partially characterised.

Which non-animal alternatives did you consider for use in this project?

Some aspects of blood cell differentiation and both normal and malignant stem cell maintenance can be mimicked in vitro, and we are pursuing these as methodologies where possible. In particular we have considered the use of in vitro cultures for the maintenance and differentiation of hematopoietic stem cells, and for maintenance and screening of malignancy-propagating cells.

Why were they not suitable?

Some aspects of blood cell differentiation and both normal and malignant stem cell maintenance can be mimicked in vitro, and we are pursuing these as methodologies where possible.

So far, we have not been able to replicate HSC fate-restriction ex vivo, using in vitro differentiation assays

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 currently performed in my laboratory, 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?

Power calculations (using the NC3R EDA and other relevant tools such as GPower) and have been used to ensure that experiments are powered adequately, and reduce the risk of significant overpowering.

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)

We have planned to source mice from central colonies where possible (e.g. C57Bl6/J mice for use as recipients) to avoid redundant breeding within 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?

Breeding strategies will be optimised to generate the lowest possible number of mice that cannot be used, by setting up crosses that simultaneously generate experimental and control mice where possible.

For mouse lines that are not in constant use we will preserve strains by sperm freezing or embryo cryopreservation, as appropriate.

For mouse lines that are commonly used we will interact with local colleagues to minimise the number of independent colonies of these strains that are maintained.

We will plan experiments within the lab, and where relevant across labs, to share tissues and cells from mice.

We will optimize our molecular and functional readouts to minimise the number of mice needed to generate input materials.

For pharmacological studies we will use pilot studies to determine effect sizes, and experimental block design to avoid overpowering the analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models used in this project are:

Mice containing constitutive and inducible fluorescent reporters: these will be used to identify specific hematopoietic cell populations and measure their output of blood cells. The genetic modifications themselves are not harmful to the animal. Administration of the drugs used to induce gene expression (such as tamoxifen, poly(I-C)) cause transient discomfort. However, these are the most efficient and inducers available, and no suitable alternative currently exists.

Mice that undergo ageing: in the study of ageing there is currently no suitable alternative to the use of intact organisms, as the processes involved are highly complex, involve both systemic and cell-intrinsic factors, and accurate replication of in vivo metabolic states. We here age mice up two years of age, as this represents the physiological age equivalent to the human age (65-70 years) where immune function declines and blood cell production is

significantly altered, where development of blood cancers accelerates, and their treatment becomes more difficult. Ageing mice to this point is therefore necessary (and sufficient) to study the effects relevant to human health. As in humans, ageing leads to physiological decline, which can cause minor discomfort; however, this is necessary in order to understand the underlying processes and devise strategies to counteract the negative effects of the ageing process.

Mice that develop hematological malignancies: The mouse currently represents the most accurate model for the experimental study of human hematological malignancies, as the disease-causing mutations can be accurately replicated and the resulting phenotypes are good representatives of the human conditions. For disease phenotypes it is necessary to allow the disease processes to proceed to a clinically relevant stage, which may cause moderate suffering for a limited time. However, this is required to obtain information relevant to human health, and will be minimised by frequent monitoring and ending the experiment as soon as an adverse clinical phenotype develops.

Mice that undergo bone marrow transplantation: The use of bone marrow transplantation represents the most refined methodology for the identification and characterization of hematopoietic stem cells and hematological disease-propagating cells, and for studying the differentiation of hematopoietic progenitor cells under physiological conditions. It is associated with transient discomfort due to irradiation where used; however, this is required for ablation of the endogenous hematopoietic cells which in many cases is a prerequisite for the transplanted cells to engraft.

Mice that are treated with pharmacological agents: To develop pharmacological strategies to counteract age-related phenotypes or to treat hematological malignancies the use of drugs is required. As ageing is a chronic condition anti-ageing compounds may have to be administered over long periods of time; however, this is necessary to determine their ability to counteract the ageing process. In addition, some drugs that are used to treat hematological malignancies are cytotoxic, and will cause transient suffering, but no lasting harm. However, their use is necessary to determine if treatments are able to improve on the current standard of care, and the least toxic alternative will be used.

Mice that are subjected to fasting: Intermittent fasting of mice is necessary to investigate the ability of metabolic reprogramming to counteract ageing-associated phenotypes and to improve therapy against hematological malignancies. This may cause transient mild discomfort, but no lasting harm (it may actually improve animal welfare, especially of aged mice).

Mice that are engrafted with human hematopoietic cells: Xenografting is the currently most refined model for studying normal human and malignant cells in a physiological setting. It does not cause any significant lasting harm, except those associated with disease progression in case of engraftment of malignant cells, as we generally use NSGW41 mice where irradiation is not required for transplantation.

Why can’t you use animals that are less sentient?

The mouse is the least sentient model with a hematopoietic system that sufficiently resembles the human hematopoietic system for the study of ageing and malignancy to be relevant to human health.

The hematopoietic system only reaches its mature state 5 weeks after birth. To obtain

results relevant to adult hematopoiesis and ageing we therefore need to use adult and aged mice. In addition, the vast majority of hematological 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?

We will use the least harmful methodology for transplantations, using sublethal or no irradiation where possible, based on pilot experiments testing the feasibility of doing so.

We will use the most accurate genetic modelling available for the study of hematopoiesis, and in particular use conditional genetics where possible to avoid adverse effects due to genetic alterations in non-hematopoietic tissues.

For experiments that involve treatment of hematological malignancies to model the clinical standard-of- care we will consult with clinical haematologists to define the least harmful treatment type that achieves clinically relevant results.

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 software 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 PILs attached to this PPL, as well as myself, will attend all of our local animal welfare meeting. I subscribe to the NC3Rs newsletter, and discuss relevant points in our weekly lab meetings
All PILs attached to this licence will be encouraged to attend the annual institutional 3Rs day, which I will also attend.

90. Genetic and environmental effects on mouse embryonic and placental development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

developmental biology, mouse embryology, birth defects, gene-environment interaction

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 investigate how genetic and environmental factors affect mammalian embryonic and placental development, concentrating on, but not limited to, the formation of the cardiovascular system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
3% of people are born with some kind of birth defect. Of these, congenital heart disease (CHD) is the most common type of birth defect, representing about one third of the total number. CHD is defined as a structural abnormality in the heart that is present at birth, and it is a major cause of infant mortality and morbidity, requiring ongoing medical treatment

throughout life. Currently only ~30% of CHD can be attributed to known causes such as genetic syndromes. This is partly because our understanding of the genetics of embryonic development is incomplete, and partly because non-genetic factors can perturb this process. Examples of non-genetic factors include maternal diabetes; exposure to reduced oxygen levels (hypoxia) due to smoking or altitude; elevated maternal body temperature (hyperthermia) due to viral infection or extreme heatwave conditions; deficiencies of dietary micronutrients such as folate or iron; and exposure to toxic substances during pregnancy. My results will provide further insights into the genetic and environmental causes of CHD, as well as other types of birth defects. This will improve diagnosis, management and genetic counselling for families with inherited birth defects. It will also help identification of the extent of the risks of environmental factors through improved epidemiological studies, assist in reducing the risks posed by these factors through improved public health policy, and perhaps even guide the development of therapies to reduce the incidence of birth defects.

What outputs do you think you will see at the end of this project?

At the end of this project we will have generated new knowledge of how both genetic and environmental factors cause birth defects. These results will be disseminated by making conference presentations and publishing papers in high impact journals. After publication, we will raise awareness of our results through the general media as well as through charities such as the British Heart Foundation and Diabetes UK. We will have also generated new resources, such as RNASeq data, that will be useful for future studies in this scientific field.

Who or what will benefit from these outputs, and how?

Our results will be of use to a broad range of academic and non-academic users:

other researchers in the fields of developmental biology and diabetes research. Our outputs will assist in a greater understanding of embryonic and placental development in both normal and pathological conditions.

clinicians and patients. Our genetic outputs will enable new genes to be added to genetic screening programs to identify if there is a genetic cause of individual cases of birth defects.

the general public and policy makers. Dissemination of our results through the news media and charities such as Diabetes UK and the British Heart Foundation, will raise awareness of environmental risks factors during pregnancy.

the pharmaceutical industry. Our work may provide a rationale for the development of therapeutic interventions to reduce the prevalence birth defects.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work we will actively collaborating with other specialist groups to extend the use of our genetic and environmental models to study their effects on aspects of embryonic and/or placental development for which we lack sufficient expertise, or time, to study ourselves. Additionally, we will to freely share these models with other groups for their own research, without any conditions.

We constantly endeavour to increase the awareness of the scientific and clinical community of our work, and the availability and utility of our animal models. This includes writing reviews (e.g. we wrote a review on the different types of environmental factors affecting embryonic heart development), giving presentations at conferences and seminars at a wide range of locations and topics, and engaging with the public through giving media interviews, writing blog posts, and participating in events (e.g. Pint of Science).

Species and numbers of animals expected to be used

- Mice: 26,000 juvenile to adult; 30,000 embryos E14.5 to birth.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 investigating how both genetic and environmental factors can perturb embryonic and placental development to cause birth defects. We are using mice because they are easily genetically manipulated , and because mouse embryos develop in a very similar manner to human embryos, and insights derived from mouse embryological studies translate well to the clinic. In addition, the genes, proteins, signalling pathways and cells involved are almost identical between mouse and human. We require analysis of developing embryos in vivo, as this process is four-dimensional (i.e. varies in space and time) and in vitro systems cannot mimic the complexity of a functioning organ such as the heart, let alone an embryo. Computer simulations can extend theoretical approaches to embryonic development, but cannot tell us about the real biological processes occurring. This work will use both wildtype and genetically altered mice. Genetic modifications will include transgenic mice with reporter genes to help detect where specific genes are normally turned on or off during the formation of the embryo; transgenic mice enabling the activation, deletion or mutation of one or more specific genes, either throughout the embryo or within particular tissues or structures in the developing embryo; and combinations of these two.

Typically, what will be done to an animal used in your project?

For two-thirds of mice, the only procedures will be breeding and maintenance (protocol 1), in which we will produce genetically-altered mice for use in protocols 2-7. The other third may be subject to additional procedures. These male and female mice will always be timed mating to produce embryos. In most cases, the resultant embryos will be analysed after death, but in less than 5% of cases these embryos will be analysed in utero by non-invasive means. In addition, female mice may be: exposed to a single environmental challenge (e.g. hypoxia, to mimic smoking or altitude; hyperthermia, to mimic viral infection or extreme heatwave conditions; or dietary modification, to mimic nutrient deficiencies) or have substances administered (e.g to activate or repress gene expression, to induce or prevent birth defects, or to allow in utero embryo imaging).

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice (over 80%) will not experience adverse effects because they will only be used for breeding and maintenance or timed matings.

No adult mice will have structural birth defects. All embryos and pups with birth defects will be culled humanely before 5 days old.

A small percentage of adult mice exposed to altered environmental conditions may suffer some adverse effects:

Alterations of oxygen levels may include transient distress similar to a human at high altitude, including lethargy and increased breathing rate at the beginning of the procedure (for approximately 30 minutes). The mice will never be exposed to hypoxia for longer than 8 hours but are not expected to show adverse effects for longer than 15 minutes. If distress persists for more than 30 minutes at the start of the exposure, the animal will be killed by schedule 1 method.

Hyperthermia may cause distress and dehydration, similar to a human sitting outside in the shade during a heatwave. The mice will never be exposed to hyperthermia for longer than 120 minutes. If distress persists after exposure, the animal will be killed by schedule 1 method.

Dietary modification may occasionally lead to weight loss over a period of weeks. If weight loss is more than 15%, the animal will be killed by schedule 1 method if humane endpoints are met.

Hyperglycaemia, similar to that of untreated type I diabetes in humans, will result in more frequent urination, and may result in weight loss. The animal’s weight (or body condition score for pregnant mice) and blood glucose will be monitored regularly throughout the protocol. In addition, cages will have extra absorbent bedding and/or will be changed more frequently, and water bottles will be changed more frequently to ensure ad libitum access.

Mice will be frequently observed for meeting defined humane end-points, but most are not expected to experience these adverse effects.

Impacts and adverse effects from the administration of substances to activate or repress gene expression, to induce or prevent birth defects, or to allow in utero embryo imaging are expected to be rare, but may include damage from administration route (e.g. gavage causing oesophageal rupture, injections into pregnant mice inducing miscarriage, wound breakdown after implantation of slow- release pellet or osmotic minipump), adverse phenotypes in response to alterations of gene expression (e.g. smaller size), or adverse effects in response to substances (e.g. weight loss). Mice will be frequently observed for meeting defined humane end-points, but most are not expected to experience these 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)?

For the mice on breeding and maintenance protocols and males for timed matings (which make up 80% of our mouse usage), we expect all mice to experience sub-threshold severity.

For adult mice experiencing hyperglycaemia, hypoxia or hyperthermia to investigate their effects on embryonic and placental development (protocols 2, 4 and 5, maximum of 1000 mice for each protocol), we expect the majority to experience moderate severity (approximately 70%) as a result of hyperglycaemia/hypoxia/hyperthermia and/or additional steps (such as blood sampling, substance administration and/or in utero imaging).

For mice experiencing dietary modification or teratogen administration to investigate their effects on embryonic and placental development (protocols 3 and 6, maximum of 1000 mice and 5000 embryos for each protocol), we expect the majority to experience mild severity (approximately 80%) and 20% to suffer moderate severity as a result of additional steps (such as blood sampling, substance administration and/or in utero imaging) combined in one animal such that cumulative suffering could be considered moderate.

For genetically altered mice to investigate the effects of gene mutation on embryonic and placental development (protocol 7, maximum of 1000 mice and 5000 embryos), we expect the majority to experience mild severity (approximately 80%) and 20% to suffer moderate severity as a result of additional steps (such as blood sampling, substance administration and/or in utero imaging) combined in one animal such that cumulative suffering could be considered moderate.

Approximately 60% of the experimental embryos (5000 for each protocol) will be collected before two- thirds gestation (not regulated), and the remainder between two-thirds gestation and 5 days after birth (maximum severity 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?

We are investigating how the specific shape and function of the heart, placenta and other organs form in the embryo. Mouse embryos develop in a very similar manner to that of humans, and the genes, proteins, signalling pathways and cells involved are almost identical. Mice are the only mammalian model to which can be applied forward and reverse genetics to assess the effects of individual loss or gain of gene function on cardiac formation. Over the past 30 years, very powerful techniques have been developed to turn on and off individual genes in the developing embryo, both throughout the embryo or just within a single organ or tissue. These have been shown through our studies and others to faithfully recapitulate many birth defects. Furthermore, gene-environment interaction studies in mouse are well documented in the literature. Embryonic development is a four-dimensional process (i.e. varying in space and time), and therefore requires the analysis of whole developing embryos. Direct genetic studies of foetal (embryonic) humans are difficult practically, and only descriptive analyses are possible, as experiments are ruled out on ethical grounds.

Which non-animal alternatives did you consider for use in this project?

Tissue culture systems and computer simulations.
Why were they not suitable?

Tissue culture systems, although they can provide useful information on certain molecular or cellular phenomena, cannot mimic the complexity of a functioning organ such as the heart or placenta, let alone the developing embryo. Computer simulations can be valuable in extending theoretical approaches to embryonic development, but cannot tell us about the real biological processes occurring in the embryo. Finally, gene-environment interactions 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?

Projected animal usage is based entirely on the experiments to meet the objectives of the PPL, with the relevant genetically altered strains either currently in house or to be imported into the colony. Most animals will be used for colony breeding and maintenance, where excess mice will be culled by a Schedule 1 method. The numbers requested are sufficient to maintain working colonies for each genetically altered mouse strain and for expansion to generate the required number of embryos for phenotyping. For example, to maintain a particular reporter or Cre strain, we will house 6-18 mice at one time, and replenished every 4-8 months. However, breeding numbers may vary depending on the specific genotypes and numbers of embryos required for a specific project. For example, to produce the mice for protocol 2, we require female mice of a certain genotype. On average, half the mice in each litter will be female; and half the females with be the required genotype. Thus, on average only one quarter of the mice from each litter will be used for procedures. The remaining mice will be culled by a Schedule 1 method after genotyping. This will necessitate maintaining a higher number of breeding pairs to get sufficient females of the correct genotype than if all females in a litter had the correct genotype.

Experiments are designed to generate statistically significant results using the minimum number of animals. For all experiments we will consult regularly with statisticians in the neighbouring Statistics Department and also with colleagues doing similar experiments to ensure we are employing the minimum number of animals to power the analyses for statistical significance. For example, in Objective 1 in order to determine the penetrance of a particular cardiac phenotype, or of embryonic lethality, a sufficient number of litters will need to be analysed, at various developmental stages, in order to correlate phenotype with genotype and to calculate Mendelian ratios. For each new strain, we will perform a pilot study initially in a small number of litters (n=5), at one or more appropriate developmental stages, for histological analysis. We will scale up for a comprehensive analysis once a phenotype has been confirmed and the most informative developmental stages have been determined.

Two types of experiment are proposed: qualitative studies without a specific numerical end-point, such as importation of new transgenic mice where a few founders will be sufficient, and quantitative analysis which addresses questions such as the role of a particular gene in normal embryonic heart development or the mechanism of perturbation of heart development by environmental factors. In the quantitative studies, two or more groups will be compared over two or more time periods with respect to continuous variables. Regression analysis will be used to quantify differences between groups. For

example, to assess the impact of a particular environmental stress on heart development, power calculations suggest that experimental groups with ~30 embryos are required to obtain 80% confidence of detecting a 10% difference in heart defect incidence.

Finally, we commit to validate experimental design using the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) “Experimental Design Assistant”.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible, genetically altered mice will be bred as homozygotes to minimize excess waste.

Animal numbers will also be minimised for Objective 1 by the fact that we will predominantly be using knockout lines from the DMDD programme for which we already know there is a heart defect.

Procedures on live animals will be used to create embryos, the final phenotyping of the morphological, cellular and molecular effects of genetic and environmental factors on embryonic development will mostly be done using fixed tissues from culled embryos and only in the case of live imaging will we require procedures to be performed on live embryos.

We have used the NC3R's Experimental Design Assistant to help design analysis of both qualitative studies of embryo morphology and quantitative analysis of changes in gene/protein expression. We have also used statisticians to advise on POWER calculations, and the relevant statistical analysis required to provide meaningful results.

Where possible we will use in vitro cell culture system to confirm the molecular findings of our studies. For example, where an environmental stressor causes perturbation of the action of a particular intracellular signal transduction pathway, we will attempt to model this in vitro to verify our conclusions.

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 put advice on efficient breeding into practice. In general, breeding cages are separated after 4-5 litters and retired at six litters.

For wild type strains shared with other labs/outside suppliers, we buy/import animals rather than generate them in-house

Where possible, mouse lines are frozen down. In cases where we do not anticipate usage in the next 6 months, lines are not maintained.

We perform breeding calculations before we plan our experiments and only produce the numbers of animals that we need.

Animal numbers are recorded on a database in real time, and are monitored regularly.

We ensure any welfare or husbandry requirements of the strain have been identified. The cage card alerts people to any special welfare or husbandry requirements.

Tissue samples are analysed for multiple different assays (e.g. by staining consecutive sections with different probes or by multiplexing immunohistochemistry on individual slides). In addition, excess tissue samples are shared with other researchers, where possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The main source of potential discomfort within this project is through the possibility of adverse effects via the use of genetically altered mice. These animals may experience adverse effects depending upon the nature of the introduced genetic material, site of insertion and level of altered gene expression.

Heterozygous breeding animals supplied from commercial sources are well characterised and documented for any potential adverse effects. The vast majority of these strains do not cause pain, suffering, distress, or lasting harm to the animals. However, in many genetically-altered mouse lines where key genes involved in embryonic development are perturbed, homozygous animals may die in utero (embryonic lethal). Homozygous animals are produced from heterozygous intercrosses.

Therefore, to minimise adverse effects, our breeding colonies will be maintained as backcrosses (heterozygous GA x wildtype), and we will only use heterozygous intercrosses to generate our experimental animals. In maintaining colonies, procedures such as tail-tipping for genotyping will only be used very rarely. In addition, the use of partially characterised mouse lines arising from the Deciphering the Mechanisms of Developmental Disorders (DMDD) programme for phenotyping embryonic lethal mice will reduce the numbers of animals used for Objective 1 of this study. In studies of gene

function using transgenic mouse models there is the element of risk that the gene selected for knockout will not have the phenotype expected. For example, knocking out a gene with very strong evidence for a critical role in heart development might still result in a mouse line without any embryonic heart defect at all. This could result in the use of significant numbers of mice for little benefit. However, in general I will use mouse lines arising from the DMDD programme that are already known to have a specific type of heart defect at the single embryonic stage analysed (E14.5), but still require further analysis to understand why they develop these defects as this has not been undertaken. This will ensure that the number of mouse lines to be imported for analysis will be the fewest possible to successfully complete the study, thus making the most efficient use of mouse numbers.

For studying the effects of maternal diabetes on embryonic development, we will use the ßV59M mouse strain. This is a recently developed model that is the least invasive diabetes model available. In this model, mice are phenotypically normal until 2 days after tamoxifen injection, whereafter they become hyperglycaemic. This minimises the time that these mice experience the adverse effects of hyperglycaemia. If required, this hyperglycaemia can be reversed by administration of sulphonylurea drugs such as glibenclamide. This is in sharp contrast with other commonly-used diabetes models such as streptozotocin-induced pancreatic ablation. This latter technique is much more invasive because mice must be made diabetic, then kept euglycaemic with implanted insulin pellets prior to mating, followed by removal of the pellets once pregnant.

For studying the effects of maternal hyperthermia on embryonic development, previous studies by other research groups have immersed pregnant mice in water warmed to 43 degrees. This is the quickest way of increasing core temperature, but mice do not like getting wet, so this causes extra distress over that caused by the increased temperature. We will use exposure to increased air temperature to increase core temperature. Although this takes longer than immersion in hot water, it is a less stressful method that also allows careful monitoring of the animal’s reactions to elevated temperature. In addition, it can take place in its home cage, with free access to water and food to maintain hydration, and so that the animal does not experience the extra distress of being removed from its normal physical environment.

Primarily, our experiments are investigating the effects of genetic and environmental factors on embryonic development. As such, in a majority of experiments, embryos will be killed by a Schedule 1 method, or other suitable means, prior to phenotyping. This will minimise potential suffering of these embryos. Mothers will also be culled by a Schedule 1 method prior to embryo collection, and will not be re-used for subsequent experiments.

Why can’t you use animals that are less sentient?

We have chosen to study a mammalian species, the mouse, so that the principles emerging from our research have the greatest chance of applying to the human situation. Mice have been used throughout scientific history and are today considered one of the best models of mammalian embryonic development. Thus, it is the most refined choice for

our studies. Less sentient animal models commonly used in developmental biology are the zebrafish (Danio rerio), the fruitfly (Drosophila melanogaster) and the nematode worm (Caenorhabditis elegans). Although all of these have a similar range of genetic tools available to mouse, their embryos all develop independently of their mother and none has a four- chambered heart. The chicken (Gallus domesticus) does have a four-chambered heart, but lacks the genetic tools available to mouse, and its embryos also develop independently of the mother. Thus, none of these less sentient animals accurately reflect human embryonic or placental development, and using these less sentient animals would reduce the clinical applicability of our results.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will not be kept beyond 15 months of age to avoid unnecessary adverse effects associated with ageing.

For subcutaneous injections we have introduced the use of a sterile ScruffGuard (https://researchdevices.com/product/scruffguard-reusable). This provides an enclosed, dark environment for the mouse which reduces its stress levels, rather than scruffing using conventional methods. In addition, more back skin is available, meaning greater accuracy for subcutaneous injections.

The cage environment is enhanced by the addition of objects that allow the animals to exhibit their natural behaviours and improve general welfare.

For subcutaneous implantation of slow-release pellets we have refined the procedure by:

cutting the superficial incision to create a subcutaneous pocket rostral-caudally as opposed to medio-laterally. This minimises the number of sutures required, makes it harder for the mouse to open their sutures as the incision site is right at the midline. In addition, there is a reduced chance of the skin stretching as the mouse moves around, meaning a reduced chance of suture opening.

The mouse is given additional cage items to aid its recovery. We provide mashed food to ensure that diabetic mice experiencing polydipsia are well-hydrated during recover and to provide a distraction to the mouse and therefore helps ensure they do not scratch their incision site. In addition, we provide additional environment enrichment, such as a gnaw stick to act as a distraction from their incision site, and a nestlet to provide extra comfort.

Where funds allow, cryopreservation of new lines will occur early in the breeding programme.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow advances publicised in the NC3R newsletter. This provides information on the most refined techniques, including new guidelines on non-aversive methods of picking up mice, single-use of needles and blood sampling. We will also adhere to updated ARRIVE guidelines on reporting work with animals as now required by many journals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend all of our local Gold Standard meetings, and will transmit relevant information to all PILs working on this PPL.

We subscribe to the NC3Rs newsletter, and discuss relevant points in our lab meetings.

All PILs working on this PPL will be encouraged to attend the local annual 3Rs symposium.

91. Mechanism and Application of FLASH Radiation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer, Therapy, Radiation, Mice

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 long-term goal of our research is to better understand the biology behind a new radiotherapy technique known as FLASH, in order to take full advantage of this promising technique for treating cancer, with higher treatment efficiency and reduced side effects. FLASH involves delivering an intense radiation treatment in a fraction of a second, which has so far only been used for one clinical treatment globally. The overall aim of this project is to 1) identifying mechanisms and optimal radiation delivery parameters responsible for an increase in the therapeutic index with FLASH and 2) expand the therapeutic application of FLASH radiation in combination with other 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?
One in two people in the UK born after 1960 will be diagnosed with some form of cancer

during their lifetime (www.cancerresearchuk.org). 30-50% of these are expected to receive radiotherapy as part of their treatment. The current level of long term (> 10 years) cancer survivors is 50% but this number is expected to increase if cancer diagnosis and treatment can continue to improve. The success of a radiotherapy treatment depends on the ability to deliver a high enough radiation dose to the tumour to eradicate it, while keeping the dose to adjacent normal healthy tissue low enough so that severe adverse effects do not occur as a result of the treatment. The quality of life following treatment is as important as the prolongation of life due to treatment. Hence, there is a need to improve the level of success of radiotherapy treatment. The most promising new radiotherapy technique is FLASH radiotherapy. In this project, we aim to better understand the biological mechanism that underpins the observed and highly beneficial FLASH sparing effect in normal tissues, to find optimal FLASH delivery parameters which maximizes the effect. Such information will be essential in our efforts to construct the next generation pre-clinical FLASH photon treatment device and to design a clinical FLASH photon treatment device, which would enable FLASH treatment of deep-seated tumours. If successful in meeting our aims, the knowledge learned will have brought us closer to a clinical implementation of FLASH radiotherapy, potentially benefiting all future patients receiving radiotherapy as part of their cancer treatment.

What outputs do you think you will see at the end of this project?

We will identify some of the potential mechanisms contributing to the FLASH effect and have a greater understanding of how it is different from conventional radiotherapy. We will also establish FLASH delivery parameters to enable the future design of clinical FLASH irradiators. We will also establish whether additional therapies have exerted an enhanced anti-cancer effect, without further damaging the surrounding normal tissue, when combined with FLASH radiotherapy. We will be able to readily translate these findings into the clinical studies currently underway (for vet practice) and in future clinical studies of human patients (treating superficial tumours with high energy electrons and treating deep-seated tumours with proton beams, e.g. brain and lung tumours).

The results of the proposed in vivo pre-clinical studies will be made available to the scientific community through presentations at National and International Conferences, and publications in peer- reviewed journals. Where applicable, the results will also be made available through publicly accessible databases, and the outcomes of this project will be highlighted through the relevant funding bodies and the 3R subcommittee as a refinement for in vivo radiation research.

Who or what will benefit from these outputs, and how?

Researchers in the international cancer and radiotherapy communities will benefit from our work, as we will present our findings through publications in peer-reviewed journals and presentations at national and international scientific conferences and meetings. Our findings will be essential when designing clinical trials with FLASH radiotherapy, with potential benefits for all future cancer patients receiving radiotherapy as part of their treatment.

In addition to the immediate professional circle carrying out similar research, we anticipate that our project will provide a cornerstone for this technical innovation in clinical radiotherapy and benefit the following groups:

The clinical research community will benefit from our results and findings, which will be

used to assess FLASH radiotherapy in clinical trials in the next 3-5 years. This will enhance the UK radiotherapy clinical trials portfolio and establish international, multi-centre clinical research collaborations.

Companies interested in commercialising relevant technologies , will also benefit from this project. The research will provide a scientific ground for new relevant technology development from the private sectors. The involvement of these commercial groups will facilitate the dissemination of FLASH technology to the wider patient population in the next 5-7 years.

Cancer patients, will benefit from the project in the longer term if the technology is successful in clinical trials and becomes a standard of care within national/international cancer management guidelines in the next 5-10 years. The quality of life of the cancer patients can be improved by the FLASH technology as less normal tissue toxicity/side effects are expected from FLASH radiotherapy.

NHS radiotherapy health care providers and the NHS will benefit from this technology in the longer term 10-15 years, as the radiation delivery will be much quicker requiring fewer medical resources. Fewer medical resources will also be required to manage the lesser side effects of FLASH radiotherapy and more emphasis can be placed on improving tumour control. The overall improvement in healthcare economics will allow the re-distribution of the medical resources to more pressing healthcare issues, benefiting everyone.

The general public will benefit from our public engagement events during this project (0-5 years). All the PIs are already heavily involved in public engagement activities organised by the Department/University. These activities will allow the general public to gain a better understanding of this state-of-art radiotherapy modality in cancer management.

How will you look to maximise the outputs of this work?

We are collaborating with other research institutes, looking at different types of FLASH irradiation (e.g. photon and proton), as well as the industrial partners with the aim of maximising the likelihood that some of the FLASH methodologies are applied in a clinical setting.

The results of this work will initially be presented to the scientific community through national/international meetings (such as annual meeting from Radiation Research Society, American Association for Cancer Research, and International Congress for Radiation Research) and appropriate funds have been requested to enable this. The meetings will be carefully selected and will include both smaller specialised conferences, as well as larger international meetings in order to reach both basic research and clinical research audiences. The data will subsequently be published in high-impact journals with relevant open access provision (such as International Journal of Radiation Oncology • Biology • Physics and Clinical Cancer Research). We will also distribute our advances in orthotopic model establishment and 3Rs to the wider research community within the UK.

We will highlight our findings on the departmental websites and where appropriate, our research will be communicated in the form of a press release linked to publication of a journal article. We will utilise social media such as twitter to publicise our findings widely and encourage post-peer review comment on our work.

Species and numbers of animals expected to be used

- Mice: We expect to use up to 2,600 mice over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice will be used in this project as their genetic background comprise of well-defined models and plenty of literatures are readily available with known reagents and treatment regimens to use.

With this project, we aim to gain understanding on the tumour response to different therapeutic strategies, specifically FLASH radiotherapy alone or FLASH radiotherapy in combination with chemotherapy (established substances), immunotherapy and/or novel compounds that will be tested under this licence. The overall goal of the licence is to identify the mechanisms responsible for the FLASH sparing effect seen for normal tissue and to find the FLASH parameters and combination treatments that will further improve the therapeutic index of radiotherapy.

It is therefore, necessary to perform this work in a living organism, given the interplay of the different components that might be responsible for the tumour response, whether within the tumour or belonging to the tumour microenvironment.

Furthermore, we are also interested in examining the response of the normal (non-tumoural) tissue to the given therapies, as this would be necessary to validate the suitability of the therapeutic strategy.

The mouse represents a feasible model to test our hypotheses, since multiple reagents, such as antibodies, are readily available, and since the current knowledge on cancer models and responses to therapies is broadly available.

Typically, what will be done to an animal used in your project?

In brief, we will induce tumours in our experimental animals and apply radiotherapy at different dose rates (FLASH and conventional) with or without a combinatory treatment, e.g. chemotherapy or immunotherapy, to analyse the tumour response to the treatment. We will also analyse the impact of these therapies in healthy tissue, to understand the effect of the treatment regimens on normal tissues.

Known agents will be administered based on literature. If novel agents are to be used in combination with radiation, we will ensure the agents have been tested safe with known treatment regimens from other PPLs.

For subcutaneous models, tumour cells will be subcutaneously implanted in the experimental animal, usually on the flank or the back, under brief recovery anaesthesia. For orthotopic models, tumour cells will be implanted locally to the intended area typically through percutaneously injection or under ultrasound guidance. Tumours will be grown for the minimum time and volume that is needed to deliver the scientific outcome. Tumours

will be measured regularly with callipers or more advanced imaging modalities.

Once the tumour reaches a certain size, radiation will then be applied to the tumours, locally, with or without a combinatory therapy, to assess the tumour response. Different substances may be given to the experimental animals before or during radiation. The preferred route would be intravenous injection to mimic the clinical condition but other route such as inhalation may be used depending on the substance.

One focus of attention in this licence is the role of the immune system in the tumour response to FLASH radiotherapy. Some experiments designed to investigate this include implantation of two subcutaneous tumours in the experimental animals. In some occasions, only one tumour will be treated, with the second tumour being used to test the systemic effect of the treatment given, in particular effects on T cell responses. This is named the abscopal effect. In other set of experiments to test the role of the immune system in the tumour response, we will aim to test that a successful therapy, manifested by tumour regression, is durable or specific to a cell line, for example. For this, we will implant a second tumour (once the first tumour has fully regressed) and observe if the tumour growth is impaired. These are the experiments of tumour re-challenge.

Experiments focused on tumour response to the different treatments, are expected to last a couple of months, generally. In some cases, experiments of tumour re-challenge upon tumour regression will be performed, and these will typically be completed within six months. Experiments of normal tissue response are completed within 3-5 days (abdomen) or within 4 months (non-whole abdominal treatment).

What are the expected impacts and/or adverse effects for the animals during your project?

The main adverse effects that the experimental animals would experience under this licence are due to:

Tumour bearing (ulcerations, intramuscular growth, difficulty to eat, pain, body weight loss).

Administration of substances, some of which might not have been previously tested in vivo in combination with FLASH radiation (toxicity, whether acute or chronic: diarrhoea, dehydration, pain, body weight loss, behavioural or neurological changes, choking or cyanosis due to technical error in gavage).

Radiation: whether applied locally to superficial tumours (skin irritation, ulceration) or to orthotopic implanted tumours or to the normal tissue (gastrointestinal and pulmonary toxicities): body weight loss, behavioural changes, pain.

Blood sampling from a superficial vein could cause bruising and damage to the tail.

Some interventions will be performed under anaesthesia, with the consequent risk of failure to recover. Best practice for anaesthesia in small rodents will be applied, and with this we expect all animals to make a rapid and unremarkable recovery from the anaesthetic.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

See Table below:

Protocol number	Title	Estimated number per protocol	Proportion of animals experience moderate severity	Proportion of animals experience mild severity
1	Normal tissue response	1000	<40%	>60%
2	Subcutaneous tumours	1000	<40%	>60%
3	Orthotopic tumours	600	<60%	>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 overall aim of this project is to understand the mechanism of FLASH irradiation on normal tissue sparing and tumour control. Unfortunately, so far the normal tissue sparing effect has mainly been demonstrated on in vivo models as little information has been known about the contribution of local tissue microenvironment on the effect. Therefore, it is essential to use animal models in the project to elucidate the mechanism. The information from animals in this project will promote the development of non-animal alternatives for future FLASH radiotherapy.

Which non-animal alternatives did you consider for use in this project?

Once we had record enough local tissue information from our project, we aim to develop relevant 2D and 3D models with relevant microenvironmental cue (e.g. oxygen level, immune infiltration etc).

Why were they not suitable?

The normal tissue sparing effect from FLASH cannot yet be demonstrated in 2D or 3D models due to the lack of local tissue microenvironment. As we are still not sure which environmental factors actually contribute to the effect, we cannot rely on the use of 2D or 3D models in our research to further develop FLASH therapy for clinical use. However, in our aim to develop relevant 2D and 3D models, we foresee that these will be able to replace animal models in future 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?

Our estimations are based on the literature on the variability of the outcomes of interest (e.g. tumour growth and outputs from functional assays). Different organ sites, tumour types, and combination of therapies will be used in our studies. These variables in our studies add to the number of animals used but is important for the completeness of our results exploring the potential of FLASH as a radiotherapy technique.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use The Experimental Design Assistant (EDA) and information from literature to aid in the design of our experiment. We have implemented additional steps to ensure randomization and blinding to avoid bias and increase reproducibility, using pilot or pre-existed data for power analysis and statistics-based guidance for future experiments.

For example, we determined the effect size from our previous experiments and the literature using the mean time to reach a specified tumour size as endpoint. This effect size was then used to calculate the sample size required in our proposed experiment to allow robust comparison between groups. This gave us the overall number of animals included in this project licence.

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 design our experiments and narrow down the study variables (e.g. radiation dose and fractionation) primarily based on the previous literatures on conventional irradiation. This will reduce our animal number in finding the dose regiment for tumour control. If transgenic mice are to be used, wild type or heterogeneous littermates with no intended phenotypes will be used as a control to minimise the breeding waste.

During the experiments, various non-invasive imaging and functional assays will be conducted on the same animal to maximise the outputs and at the end of the procedure, various tissues and blood will be collected for further investigation.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used as they are the best characterised species for the types of experiments we propose in this project licence. Immunodeficient mouse strains will typically be used, with naturally occurring or genetically engineered mutations. In addition, immunocompetent mouse strains with pre-existing mice cancer cell line will be used in this project.

For subcutaneous models, tumour cells will be subcutaneously implanted in the experimental animal, usually on the flank or the back, under brief recovery anaesthesia. For orthotopic models, we will implant tumour cells percutaneously to the intended area by less invasive methods without suture or bone incision. Ultrasound guidance will be used to precisely position the needle for the organ not externally visible. Tumours will be grown for the minimum time and volume that is needed to deliver the scientific outcome. Tumours will be measured regularly with callipers or by more advanced imaging modalities. In some cases, where there is more than one tumour inoculated, the total tumour volume limit for a single tumour will be applied for the combination of both tumours to minimise suffering due to tumour burden on both sides. These will be carried out under anaesthesia with appropriate contrast agent when necessary.

Known agents will be administered based on literature. If novel agents are to be used in combination with radiation, we will ensure the agents have been tested safe with known treatment regimens from other PPLs. Based on our 3 years of experience with dietary modulation, during which no adverse effect on the animals have been observed, we do not expect this to have a negative impact on the animals.

Why can’t you use animals that are less sentient?

We will use adult mice as most radiation studies have been conducted on this model, which will help us with methodology and interpretation of our results. Mice are considered as the lowest order species in which we can study tumour growth and normal tissue repair in a way that is still relevant to human cancer treatment. Zebra fish is an example of a less sentient species that could potentially be used instaed of mice in some of our future studies, once we have acquire more expertise in the area. We will use terminal anaesthetised mice in some experiments to minimize animal suffering but due to the time needed for cell regeneration and tumour response, we cannot use it exclusively.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For each step in the protocols we have introduced monitoring and controlling measures to minimise the harms for each animal used in this licence. Some of the most significant ones

are specified below:

Orthotopic tumour implantation: Only methods without suture or incision will be used to generate the models.

Tumour growth and physiology: Tumour induction will be done under brief recovery anaesthesia, which will help with precise positioning of the tumour to avoid the tumour invading the underlying muscle.
Tumours will be grown for the minimum volume scientifically needed. If tumour develop wet ulcer, mice will be killed. Some tumours may develop into an ulcer, which will be monitored for signs of healing for 48h and killed if there is no improvement.

Administration of substances: Known agents will be administered based on literature. If novel agents are to be used in combination with radiation, we will ensure the agents have been tested safe with known treatment regimens from other PPLs. Only substances, administration routes and formulation that are shown not to cause adverse effects will be used for tumour response experiments. Mice will be regularly monitored and killed at humane endpoints to ensure keep the suffering to minimum.

During application of ultrasound for drug delivery, mild heating of tissue (1-4 degrees Celsius) may occur. To ensure minimal heating to the tissues, the acoustic characterisation of ultrasound will be carefully studied in vitro for the heating capacity and other PIs with relevant in vivo experiences will be consulted.

Radiation: Radiation will be applied, under anaesthesia, locally or regionally, with shielding of the rest of the body. This will minimise the parts of the mice exposed to radiation and, therefore, will avoid unnecessary harm to normal tissues.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will be based on the “Guidelines for the welfare and use of animals in cancer research” (British Journal of Cancer, 102, 1555-1577, 2010). Additional literature includes “Preclinical formulations for discovery and toxicology: physicochemical challenges” (Expert Opin. Drug Metab.
Toxicol. 2, 715-731, 2006) for administration of substances; “The Design of Animal Experiments: Reducing the use of animals in research through better experimental design” (2nd edition, 2016) for experimental design; “Handbook of Laboratory Animal Management and Welfare” (2003), the termly newsletters from the university’s 3R subcommittee and other guidance from NC3Rs. Imaging of cardiac physiology will be conducted based on “Guidelines for measuring cardiac physiology in mice [34].”

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As a general measure, we will continuously revise our methodology and adapt it according to new policies, technologies and approaches aimed at minimising welfare costs. To achieve this, we will attend the seminars provided by NC3Rs, keep up to date with NC3Rs recommendations via its website and seek for advice to improve the 3Rs in our experiments, and together with the university’s 3Rs subcommittee of the Committee on Animal Welfare Ethical Review Body (AWERB), to help diffuse our particular advances on the 3Rs.

92. Developing gene therapies to treat neurodegenerative diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Neurodegeneration, Motor Neuron Disease, Dementia, Gene Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
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 project will assess the viability of potential new therapies that may alter the activity of selected genes to stop or slow the progression of neurodegenerative diseases such as motor neurone disease, frontotemporal dementia, Parkinson’s Disease, Huntington’s Disease and Alzheimer's disease. It will help us test the feasibility, efficacy and safety of these therapies and how they may be translated to develop gene therapies 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?

There are currently no effective treatments that significantly alter disease progression for the neurodegenerative disorders listed above. As our population ages the impact of caring

for people affect will increase dramatically and this presents one of the greatest social and financial challenges to our society.

One major barrier to the successful development of new treatments is our ability to target the brain, which is challenging to access. This project focuses on gene therapy, which offers us a new and powerful tool to directly target genes that may be involved in the disease process. Doing this, a single acute intervention will have long-term effects on the disease.

What outputs do you think you will see at the end of this project?

We hope that one major output from this project will be new gene therapies for neurodegenerative diseases that can be progressed to clinical trials. In addition, we anticipate generating new knowledge regarding the contribution of various genes/proteins to the neurodegenerative disease process, and their potential to provide future targets for gene therapy.

Who or what will benefit from these outputs, and how?

In the short to mid-term, over the course of the project, the development of gene therapies that can be moved into clinical trials will deliver new hope to patients and families dealing with the relevant neurodegenerative disorder, as well as medical staff involved in the treatment of these patients. Longer term, beyond the end of this project, a successful outcome in clinical trials, which will be well predicted by these robustly run preclinical assessments, will deliver huge social and economic benefits both country and world-wide.

Additional short-term benefits throughout the course of this project will be seen by other researchers in the field of neurodegenerative diseases, as we will provide information to improve our understanding of what goes wrong in these diseases and identify new targets and approaches to try to slow or stop disease progression. Longer term, again over the course of this project and beyond, data obtained in this project may help us to identify additional targets for new therapies that can be developed to treat various neurodegenerative diseases.

How will you look to maximise the outputs of this work?

The development and validation of gene therapy vectors in this project is to be done in collaboration with a gene therapy start up company co-founded by this PPL applicant, who’s primary goal is to deliver viable and targeted gene therapies to the clinic. A structured process is in place to facilitate this, and to maximise the potential benefits of any viable new therapies developed.

While successful new viral approaches will be patented, all basic discovery data obtained in this work will be widely shared throughout the academic community, with the goal of publishing all findings, positive or negative, in relevant scientific journals. As new findings are obtained, collaborations with both local colleagues and those further afield will be sought out to further develop our research. In addition, efforts will be made to engage with the local lay community to share any findings of interest, and in cases of specific interest to patient groups, we will liaise with the appropriate support group to try to disseminate the relevant information widely and accurately.

Species and numbers of animals expected to be used

- Mice: 10,000
- 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.

This project is designed to evaluate potential new treatments for neurodegenerative diseases such as motor neurone disease and dementia. These diseases affect the brain and spinal cord as we age, so in order to test out whether new therapies might be effective in human patients, we need to be able to show that they have an impact on the progression of the disease. In order to fully assess the benefit of our therapies, we will need to study them out into old age, hence some mice will be aged. The mouse has been selected as they possess a brain region called the cerebral cortex, which is only found in mammals, and this region is preferentially affected in many neurodegenerative diseases. Most animals in this project will have genetic alterations that are known to cause neurodegenerative diseases in humans, these animals will develop aspects of the disease as they age, and are needed so that we can see if our treatments are effective at reducing or preventing the development of these disease features.

Typically, what will be done to an animal used in your project?

The work planned in this project falls into three main categories:

Normal mice will receive a brain injection of viral particles that have been engineered to deliver a possible disease treatment. This will allow us to develop the most effective delivery system possible. Following this injection, most mice will be kept for 4-8 weeks, with approximately 10% being kept out to a maximum of 2 years old, then they will be culled for tissue collection.

Normal mice will either be genetically modified, or they will receive a brain injection of a virus that contains a gene that is know to cause a neurodegenerative disease. They will then be aged out to a maximum of 2 years old, and during their life 30-40% of these mice will undergo behavioural tests to look at how well their brain works. These tests will include tests of memory, and tests for mobility. In most cases, memory tests will be done every 2-3 months, while mobility tests will be conducted monthly. A small number of animals (10%) may also receive brain scans to look at their brain function. At the end of the testing period, mice will be culled and tissue collected.

Mice that model a neurodegenerative disease will receive a potential therapy or a placebo either via brain injection (80% of animals) or via a drug given orally or as a peripheral injection. They will then be aged out to a maximum of 2 years old, and during their life 30-40% of these mice will undergo behavioural tests to look at whether the treatment is working. These tests will include tests of memory, and tests for mobility, and as above, in most cases, memory tests will be conducted every 2-3 months, and tests of mobility will be conducted every month. A small number of animals (10%) may also receive brain scans to see if the therapy has improved their brain function. At the end of the testing period, mice will be culled and tissue collected.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that undergo brain injections may experience some discomfort in the first 2-3 days following the injection, but this is not expected to have any long-term consequences.

Animals that model a neurodegenerative disease are expected to develop either mobility or memory problems as they age.

Memory problems are not expected to have major impact on mouse welfare, although a small number of animals may display reduced self-care as the disease progresses. If this results in significant discomfort or distress to the animal that cannot be rectified by providing food and water that is easy to access, the mouse will be culled.

Mice that develop mobility problems are expected to experience mild to moderate difficulties in moving around their home cage. These difficulties are not associated with any pain. Mild impairments are not expected to cause any significant distress or suffering to the animal, and these mice may be kept for several months. If the motor impairment worsens, animals will be given moistened food at floor level, to ensure they can successfully feed. If they show any evidence of a decline in general health, they will be culled.

Animals on this project that are maintained out to old age may develop various age-related issues that are common to mice, such as benign tumours or hair loss, and in some cases sore patches on the skin. If these effects are only mild, and cause minimal distress, the animal may be maintained for several months, however, if any of these issues are thought to cause significant pain, discomfort or distress, the animal will be culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 75-80% of mice on this project are anticipated to undergo brain injections, which will cause short-term moderate discomfort, without any long-term welfare issues. Disease model mice will form a major component of this project, and are expected to make up 60-70% of total animals used.
Approximately half of these are only expected to develop mild memory problems, while the other half are expected to develop mobility issues that will cause mild to moderate discomfort as the animals age. Since most of these animals will undergo a brain injection to try to treat their disease, most mice on this project (75-80%) are expected to fall into the moderate severity category. All other animals are not anticipated to exceed a 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?

This project aims to test new therapies that may be effective in treating neurodegenerative diseases, and in order to fully establish whether these treatments will be effective in humans, we need to see whether they can slow or stop the progression of the clinical features of the disease, which can only be done by using live animal models.

Which non-animal alternatives did you consider for use in this project?

Various cells, including stem cells (grown in a dish).

Brain slice cultures (thin slices of brain that are grown in a dish).

Why were they not suitable?

A major aim of this project is to see whether our potential new treatments improve the memory and movement problems patients suffer with in neurodegenerative disorders like motor neurone disease and dementia, which can only be assessed in living animals. We also want to see how the treatments may alter the progression of the disease in the brain as it ages, and at present, none of our non-animal models can fully mimic the impact of interactions between the millions of cells within the brain, and also how communication with, and the function of other parts of the body can impact on the brain, and how these diseases progress.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 to be used on each protocol of this project have been estimated as follows:

For the assessment of new genetically modified mouse lines, or other disease models, numbers have been selected based on our experience with pre-existing mouse lines that are expected to behave similarly, along with an estimation of the number of new genes or gene variants we are likely to want to investigate over the course of the project.

For the validation and testing of new viral delivery systems, numbers have been estimated based on total numbers required for the validation of each system, together with an estimation of the number of different variants we expect to be testing over the course of the project.

For therapy assessment, numbers have been estimated based on total numbers required for the validation of a single therapy or therapeutic target, together with the total different number of disease models and therapies we are hoping to test over the course of the project.

In addition to this, we have accounted for the number of animals required to maintain our different lines of genetically modified mice throughout the course of this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the design phase of each experiment, appropriate searches were done to identify what mouse lines may be available for us to use, and to ensure we had access to all relevant information regarding expected outcomes in these animals. Wherever possible, lines to be used were chosen based on how easily we would be able to measure the impact of our therapies on the disease. Only if no suitable lines were identified, were plans made to generate additional models of disease.

Consideration was also given to how much variation there is between control animals for each of the factors being measured, based on our own experience as well as reports in the literature. Extra consideration was made for a number of variables we control, including sex, age and background strain. The statistical approaches to be used at the end of the study formed a major part of the experimental design process, ensuring that all experimental approaches are robust.

The experimental design was also discussed with other researchers familiar with these kinds of studies, to further validate the design, and to ensure that we had included adequate control groups to each experiment, such that all data obtained will be valid.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As far as possible, all experiments will be conducted longitudinally, where we will monitor the same animals throughout their life, to allow us to maximise the data obtained from each animal. For tissue harvest, all organs of interest will be harvested from each animal, and experiments will be designed to allow multiple follow ups in the tissue from each animal, thus minimising the total number of animals required.

Where relevant, and in most case of therapeutic testing, small scale pilot studies will be conducted prior to each full scale study, with follow up analyses to ensure that the treatment appears to be working as predicted, without any unexpected adverse effects, and also to allow subsequent experimental design to be optimised to use the minimal number of required animals. This will ensure that unnecessary large scale experiments do not take place.

As far as possible, breeding strategies will be designed so that all animals from a mating are used for an experiment, and for general maintenance of a line, animal breeding will be monitored and controlled to ensure that we obtain sufficient mice to maintain the line, while minimizing the birth of mice that are not 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.

Almost all the work planned in this project will use mice. These include healthy control mice, and mice that contain genes that give them aspects of various neurodegenerative diseases such as motor neurone disease and dementia. Since these are all diseases of ageing, all animals are healthy when they are young, and develop memory or movement problems as they age. None of these models result in any pain for the animals, but some animals will develop mobility problems. These problems are progressive, and so animals can be monitored for any decline, and additional care measures can be implemented to minimise distress if required. Experiments will be designed to ensure that these animals do not experience any significant long term discomfort as a result of these movement problems, and in most cases, disease models that only develop mild mobility issues will be used.

Injection into the brain under anaesthesia is a procedure that is required for many of our therapy treatments to ensure the treatments reach our target sites. This procedure is minimally invasive, and animals are usually completely recovered from this within a week.

A small number of healthy control mouse and rat pups will be killed to provide brains for cells and brain slice experiments in place of live animals wherever this is possible. The use of this model will ensure that animals do not experience any distress or suffering as a result of the experimental aims.

Why can’t you use animals that are less sentient?

This project is designed to assess how well new therapies will work to treat neurodegenerative diseases like motor neurone disease and dementia. These are diseases of ageing, which result in impairments in memory or mobility. We therefore need to be able to assess whether our proposed treatments can improve these clinical symptoms. In order to do this we need to be able to work with models which allow us to measure these behaviours as the animals age, to see if disease mice receiving the treatment do better than those that do not.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals undergoing brain injections will be given a long acting pain killer and local anaesthesia to minimise surgery associated pain. They will be monitored for at least one week following the surgery to check for any evidence of pain, and if required, will be given additional pain killers.

Disease model animals that develop movement problems that may impair their ability to access food and water will be given moistened food at floor level, and will weighed at least weekly (daily as required) to ensure they are remaining healthy.

Any animals undergoing frequent handling as a part of the experiment will be handled regularly prior to the start of the experiment, to ensure that this process does not induce unnecessary anxiety or stress to the mouse. In addition, all mice will receive appropriate training for all behavioural tasks prior to the onset of experiments, both to minimise anxiety

and stress, and also to improve outcomes, which should ensure consistent data from all mice, and hence allow the use of the smallest number of tests and animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All work will be conducted following the general principals of the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly check the NC3Rs website for updates, as well as liaising with both our NTCOs and our local NC3Rs regional programme manager. Any relevant changes will be implemented immediately for all new experiments, and consideration will also be given to implementing changes to ongoing experiments, provided any such change is not expected to have an impact on the animals such that it may alter research outcomes.

Home Office

93. Transgenic Mouse model for poliomyelitis

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

Transgenic mouse, Poliomyelitis, Polio vaccines, Safety, Efficacy

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 determine the safety and efficacy of poliomyelitis vaccines as well as antiviral treatments against poliovirus infection

To evaluate the effect of different genomic mutations on the attenuation phenotype of poliovirus strains and variants

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 Transgenic mouse neurovirulence test is an important vaccine safety test for Oral Polio Vaccine (OPV). The test in transgenic mice carrying the human receptor for

poliovirus has largely replaced the test in non-human primates that were previously the only adequate models for testing vaccine safety. This transgenic mouse assay has been established by the World Health Organization (WHO) through collaborative studies and is detailed in WHO and European Pharmacopoeia regulatory documents, which specify the procedure, numbers of animals and the particular line of mice to be used. They have been designed to minimise animal usage and suffering while producing statistically reliable results.

The production of OPV has decreased but is still an important tool for outbreak control, so OPV production will still continue for several years until polio is eradicated globally. It is possible to produce batches of vaccine that fail the test, and in rare instances the vaccine can cause poliomyelitis itself.
Thus testing of every batch is necessary and currently there is no alternative to the use of animals although the developmental aspects of the work to be undertaken aim to explore alternatives based on molecular biology which will be validated against the mouse test.

The test will also be used to qualify new attenuated strains to be used for vaccine production as part of the global polio eradication final stages.

Finally, there is rising interest in the use of inactivated polio vaccine (IPV) and other non-infectious vaccines including the use of new strains and antiviral compounds whose effectiveness needs to be evaluated. The mouse model provides a way of assessing protective efficacy and will be increasingly important for this purpose.

What outputs do you think you will see at the end of this project?

We will generate scientific information that will allow us to further improve quality assure methods which supply vaccines of high quality and safety and hopefully lead to a significant reduction of the use of non-human primates for testing vaccines globally. We will further investigate the effect of different poliovirus mutations on its attenuation phenotype to validate molecular methods against tests in mice to further reduce the need of animals for vaccine testing. Importantly, we will use results from tests in mice to support the clinical evaluation of new poliovirus vaccines which will be essential to complete global polio eradication. We aim at publishing our findings in peer-reviewed scientific journals.

Who or what will benefit from these outputs, and how?

Continuing to be able to perform the test in the medium to long term (3-10 years) is essential to maintain the ability to effectively control the release of OPVs and facilitate vaccine development globally.
Vaccines being assessed by these quality assured methods are essential to the continued success of the polio eradication programme which depends on the supply of vaccines of high quality and safety. New strains being developed and tested in clinical trials also provide more benefits to the polio eradication programme.

The reduction in the use of non-human primates supports 3Rs initiatives. Results obtained with this test can be compared to those from other possible assays, particularly laboratory tests not requiring the use of animals, giving greater confidence in their reliability, applicability and validity for the batch release of vaccines. Therefore, the benefits of this work will have ramifications at the national, European and global level.

How will you look to maximise the outputs of this work?

Continued collaboration is essential as part of this work, international workshops with all institutions involved in the testing, including WHO, are held annually which means discussions on improvements and refinements to the testing can take place. There are also grant-funded projects which provide opportunities for collaboration and discussion with all parties involved in the development of novel strains of OPV. Projects looking at replacement of animal test with next generation sequencing are also in process which will provide valuable data for 3Rs initiatives.

Species and numbers of animals expected to be used

- Mice: Up to 30,000 mice are expected to be used in 5 years (25,000 bred at our Establishment).

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 test in transgenic mice carrying the human receptor for poliovirus has largely replaced the test in non-human primates that was previously the only adequate model for testing vaccine safety. This transgenic mouse assay has been established by WHO through collaborative studies and is detailed in WHO and European Pharmacopoeia regulatory documents, which specify the procedure, numbers and life stages of animals and the particular line of mice to be used. They have been designed to minimise animal usage and suffering while producing statistically reliable results.

Typically, what will be done to an animal used in your project?

Genetically modified mice are bred under one protocol in this licence under strict conditions laid out in the project.

Suitable animals will be selected and randomised to meet the scientific requirements. This will be done at an early age and maintaining original weaning groups where possible to minimise aggression between males. Husbandry measures will be adopted to minimise any aggression. The animals will be monitored closely for signs of aggression and animals separated if needed.

Animals will be inoculated with different biological preparations using different inoculation routes. In case of intraspinal inoculation, a surgical procedure is performed to expose the tissues around the specific area of the mouse’s spine to ensure accurate inoculation. This is done under anaesthesia and analgesia is routinely provided to all animals before the procedure. The surgical wound is closed after inoculation.

Animals might be immunised with polio vaccine or treated with an anti-viral compound before poliovirus infection. Blood may be sampled at different steps to measure anti-poliovirus antibodies. Animals may be kept for up to 12 months to investigate the persistence of antibody response and/or possible non- acute effects of the procedure and then challenged with paralysing doses of poliovirus using different routes. Infected animals will be monitored for clinical signs of polio, typically for up to 21 days after inoculation.

What are the expected impacts and/or adverse effects for the animals during your project?

Experience has shown that the genetically modified lines to be produced under this licence have no adverse effects on the mice and that the genetically modified traits have no adverse effects.

A small number of animals (less than 1%) may display trauma arising from the inoculation procedure. Affected animals will show varying degrees of limb paralysis but will otherwise appear and behave normally. There is a small risk of infection developing at the closed incision site and/or animals showing injection trauma after inoculation. Regular monitoring ensures affected animals are treated quickly and/or euthanized should their condition not improve.

After infection with poliovirus, typical clinical signs of polio start with weakness of the hind limbs, which may develop into partial or full paralysis of one or both hind limbs. There is no evidence of pain associated with the progression of polio symptoms which are rather characterised by loss of neural sensation. The degree and duration of any clinical signs arising from the virus inoculation to allow the animals to be conclusively assigned to a treatment group are not expected to exceed the moderate category due to the controls and humane endpoints in place. The endpoint will be full paralysis of one hind limb, however for some studies this will be refined to two consecutive days of partial paralysis of one hind limb. Animals reaching the required endpoint will be killed immediately. However, a small number of animals, approximately 1%, may experience a rapid onset and/or progression of paralysis that cannot be predicted, which may, in extreme cases, result in quadriplegia. Any animal showing clinical signs affecting one or both front limbs will be killed immediately regardless of whether the hind limbs are affected. There may also be cases of complications relating to the virus infection including urine retention. Animals with any clinical signs of polio will be monitored more frequently, which may include additional timepoints during the working day and outside of normal hours, to ensure the endpoints can be applied effectively.

Animals that are unable to readily feed or drink as a result of their condition will be killed immediately. Veterinary advice will be sought in all other cases of variation from normality.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected level of severity of transgenic mouse test ranges from mild to moderate depending on the protocol. Based on 2020's figures, the proportion of mice reaching sub-threshold, mild and moderate severity was 30%, 12% and 58%, respectively.

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 transgenic mouse neurovirulence test is well defined in regulatory documents. It is intended to replace the test in non-human primates and has been validated for this purpose which has helped significantly reducing the need for non-human primates for OPV testing.

Which non-animal alternatives did you consider for use in this project?

Molecular tests have been developed to measure the proportion of a mutation associated with a virulent phenotype at certain positions in the viral genome. The test is included in regulatory requirements but has still not been shown to provide the same level of information as animal tests. The advent of deep sequencing technology makes it theoretically possible to scan the entire genome and establish variability at certain positions with effect in virulence, or to demonstrate their value as markers of manufacturing consistency and differences between producers. This approach will be pursued in parallel with the in vivo based tests; if validated appropriately, in part against them, it could ultimately lead to the disappearance of the need for animals for testing vaccine safety as “safe” whole-genome genetic profiles could be established.

Why were they not suitable?
Molecular tests described above could replace some aspects of the work, particularly testing vaccine safety. However, the inherent complexity of the vertebrate immune system precludes the use of non- animal models for the assessment of vaccine-induced immunity. Similarly, there are not non-animal alternatives to study how mutations affect virulence as this requires assessment of specific clinical signs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 design of the regulatory test was based on intensive statistical discussion and input to define the minimum number of animals required to give valid results. They are detailed in official regulatory documents. With respect to the developmental work, statistical input is sought as part of the process of experimental design to ensure that the minimum numbers of animals are used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

There is full control over the training process for this technique, which means that operators can be selected based on genuine experience of proficiency in Regulated

Procedures, and can be monitored closely and constantly by experienced operators to determine genuine competence. This will help reducing the number of invalid tests and the unnecessary repetition of procedures. We will make efforts to reduce waste, promote animal alternatives (all the three Rs), and increase the reproducibility of research and testing by following PREPARE guidelines when designing experiments (https://norecopa.no/prepare). We will also follow ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments), recommendations to improve the reporting of research involving animals – maximising the quality and reliability of published research, and enabling others to better scrutinise, evaluate and reproduce it.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The number of animals used under the breeding protocol will be minimised through effective breeding colony management. Experiments will use an equal number of both sexes to minimise the numbers used under the breeding 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 only animal alternative to the mouse is the non-human primate: work to develop the mouse model has allowed the replacement of primate testing. The particular strain of mouse most commonly used is required as all validation work was conducted using this strain. This programme will also demonstrate the utility of mouse strains other than the strain used in the regulatory test.

Why can’t you use animals that are less sentient?

Our experience and that from other organizations that have implemented this test, strongly indicates that it is not possible to use less sentient animals, this testing has been validated thoroughly and no alternatives have been deemed suitable.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During husbandry and the frequent clinical scoring, the animals will be handled using refined techniques e.g. use of tunnels or cupping in the palms to minimise stress.

Improvements in technique have reduced the likelihood of complications arising from wound closure. There is a remote possibility of bacterial infection, but this has not been observed previously. Good aseptic technique will be used during the procedure. Good post-operative care will be provided including analgesia. Supportive measures will be provided during the recovery phase including the provision of additional warmth. Animals

will be examined following recovery from anaesthesia and the site monitored for any deterioration on a daily basis. Veterinarians will advise on surgical techniques and best practice.

Following infection, the animals will be provided with longer bottle spouts and diet on the floor of the cage for ease of access. Post infection, animals are examined daily in the course of the studies and clear humane endpoints have been defined. For some studies it will be possible to refine the endpoint further depending upon the scientific information that is required. The period of monitoring clinical signs will be extended beyond normal working hours as required to make sure any harms to the animals are minimised, through effective application of the defined endpoint.

Additional supportive husbandry measures will be used to ensure the animals have easy access to food and water.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The general LASA guidance is followed: Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Training protocols were optimised under WHO supervision through experience in different laboratories and discussions in training workshops.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Staff attend conferences on these subjects and are continually researching new methods. Having access to a local Animal Welfare and Ethical Review Body (AWERB) also helps in conducting regular reviews of procedures and control of best practices. Annual meetings are organised for this forum where presentations are given on 3Rs initiatives and how these have been implemented.

94. B cells in tumour immunity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

B cell, cancer, vaccine, autoimmunity

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?

Characterising the function of B lymphocytes in immunity to tumours. Developing a new tumour-specific vaccination approach that activates B lymphocytes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 main cause of death of the aged, but also of younger patients. New and cost-efficient therapies are clearly needed. Recent major advances in cancer immunotherapy have shown that the immune system is able to attack and destroy cancerous tumour growth. Still, harnessing the immune system to specifically attack structures on tumour tissues is a major challenge. Tumour tissue is made of the patient’s own tissue, i.e. it consists of immunological self-structures (or autoantigens). The problem with inducing immune responses to self-structures is that our immune system is very good at avoiding attacking immunological self in order to avoid autoimmunity. This phenomenon is called immunological tolerance to self.

A now established therapy against cancer is that monoclonal antibody drugs are injected

that target human self-antigens expressed by tumours. These antibodies are originally generated in animals. In animals these human antigens are foreign, so self-tolerance is not an issue and antibodies are produced after vaccination. Generating monoclonal antibody drugs, and the production, storage and administration of monoclonal antibody drugs are expensive and laborious, and antibody drugs last in the patients only for a limited time.

We plan to harness vaccination in order to let patients generate their own antibodies against self- structures on the tumour. Advantage of vaccination is that it is a well-established method against pathogens. Vaccines can be generated and administered cheaply and therefore have been used widely for therapy and prophylaxis in underdeveloped parts of the world. While vaccination against self- antigens should be difficult, as immunological tolerance prevents responses against self, we have developed a new vaccine conjugate that should be able to overcome self-tolerance and efficiently induce self-specific and tumour specific responses.

In the current project we plan to develop and test variations of this vaccine. At the same time, we plan to study interactions of B cells – the cells that produce antibodies to tumour structures – with tumour tissues, and the role of immunological self-tolerance in the interactions of B cells with tumour tissue.

What outputs do you think you will see at the end of this project?

The project will generate better understanding on how B cells interact with the environment in tumours (tumour cells and immune cells). Outputs will be presentations at scientific conferences, publications in scientific journals, and/or patents.

The second aim of this project is to optimise our vaccine design that is able to induce antibody responses to self-structures expressed on tumour tissues. We plan to generate variants of this vaccine and test their efficiency in pre-clinical animal models. Outputs during this project will be presentations at scientific conferences, publications in scientific journals, and/or patents. In the long term the project shall lead to the development of new vaccines that will allow preventative or therapeutic vaccination to cancer.

Who or what will benefit from these outputs, and how?

In the short term, this project will have broad local benefit, provide new information on how antibody responses are triggered to self-antigens after vaccination, and new methods how of inducing self-antigen-specific antibody responses. Mouse models of tumour development will provide information of B cells in primary tumours and tumours after vaccination, which will increase our fundamental understanding of B cell activation in different tissue environments, and in response to the tumour or to vaccination.

Further, it will let us test new versions of our cancer vaccine and its prophylactic and therapeutic efficiency against tumour growth in mouse tumour models.

In the medium to long term this project will impact on clinicians and patients: If translated into clinical use, this vaccine has potential benefits for the prevention or treatment of cancer by vaccination. Cancer vaccines have the potential to bring major benefits in treating antigen-expressing tumours, either alone, or as adjuvant or in combination therapies, to improve cancer treatment. This approach also has potential benefit for other disease types where self-antigens might be targeted.

Commercialisation would benefit UK biotech industry in the long term (>10 yr).

How will you look to maximise the outputs of this work?

This interdisciplinary project links groups working in immunology, cancer immunology, tumour biology, and clinical cancer medicine. We will present findings locally through shared meetings, and in our animal facility. This large base of scientists and clinicians interested in immunotherapy of cancer will provide opportunity for interdisciplinary discussion and project support from the local community, maximising the opportunities for new collaborations.

Results of the work will be published in high quality open access journals according to the ARRIVE guidelines and presented at both national and international scientific meetings, and the local institute websites regularly updated. We plan to publish unsuccessful approaches as well.

New approaches may be patented, which will maximise the potential to translate this into successful biotech products that become quickly available at large scale.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used in this project as preclinical models, studying the complex interplay of the local immune environment in tumours and lymphocytes, that are activated in lymphoid tissues distant from the tumour. At present, there are no models that replicate the complex interplay of different immune cells and stromal elements in tumours and lymphoid tissues in vitro.

The adult stage will be used as the antibody immune response is only fully developed in the adult. Further, immune tolerance to self-antigens will be studied, and this is only fully developed in the adult organism.

Mice are the most appropriate animal, because as mammals their immune system has a huge degree of homology with the human immune system, their immune system is best studied, and there is the largest number of experimental tools available to manipulate and analyse their immune response (e.g. gene manipulated animal strains, antibodies to detect specific proteins, and gene expression detection reagents).

Typically, what will be done to an animal used in your project?

Subcutaneous tumour models will be used in this project.

Tumour cells will be injected once subcutaneously on a single flank site. Tumour sizes will

be measured regularly.

Vaccines will be injected prior to or after the tumour implantation via subcutaneous injection.

Blood sampling may be taken during the tumour growth from peripheral blood vessels in accordance with LASA guidelines.

After not more than 6 weeks the animals will be killed by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Implantation of tumour cells under the skin may cause adverse effects. The adverse effects are expected to be transient and mice are expected to return to normal behaviour within 2 hours of implantation.

Tumour implantation may be done under short general anaesthesia. Mice will be appropriately monitored during anaesthesia. Mice will return to normal behaviour after waking up from anaesthesia.

Growth of tumour cells under the skin in some mice may cause adverse effects such as distress or pain leading to weight loss. The animals will be closely monitored after the implantation. They will be humanely killed if any adverse effects are observed which exceed moderate severity (including weight loss (maximum of 20%), a body condition (BC) score of <2.5, reduced activity, continuous hunched posture et al). Subcutaneous tumours will be measured maximum length x maximum breadth in mm. These two measurements will be used to estimate the tumour volume. If this exceeds 1.25 cm^3 then the animal will be killed.

Treatment with vaccine (e.g. immunogens, adjuvants). These may have side effects. The type of immunogens and adjuvants proposed for use in the project generally have few side effects, however we will use previous experience and data in the scientific literature, plus pilot studies to inform our 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)?

The expected severity level in all animals is moderate. The majority of animals will have a tumour implanted, and the majority of these will be vaccinated. A minority may encounter vaccination only in order to study the immune response to self-antigens.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Aims of this project are to test the role of B cells in tumour immunity, and developing a therapeutical vaccine targeting tumour blood vessel development, inhibition of tumour growth and spread. This is going to develop data to support clinical trial. At present, there are no models that can mimic the complex interactions of the immune system (lymphocytes and lymphoid organs where lymphocytes are primed) with tumours, or the development of an immune response to a vaccine in vitro. Therefore we are dependent on mouse models.

Which non-animal alternatives did you consider for use in this project?

In silico analysis of interactions of the immune system with tumours and of vaccine responses

In vitro B cell activation

Analysis of immune responses in human tumour tissue

Review of the scientific literature

Why were they not suitable?

We have been developing in silico methods to analyse immune responses, however, these are only able to test hypotheses that have been developed with data derived from in vivo experiments. They are able to inform about optimal design (e.g. optimal time points or doses) of future in vivo experiments and generate new hypotheses, but these new hypotheses have to be tested again by in vivo experiments.

Wherever possible, we do in vitro experiments to study individual steps of B cell activation and differentiation in vitro. In vitro models are useful for variation of simple processes immediate after B cell stimulation, however, do not replicate the immune system and the complexities of immune cells interacting with each other and their environment, i.e. in the tumour or in lymphoid tissues.

We are collaborating with local colleagues who do analyse fixed explant tissues from tumour patients. However, this is not sufficient to analyse the complex interactions during the initiation of an immune response, interactions between the tumour and local lymphoid tissues where the response is started, or model the complete anatomical, and cellular interactions.

We do continuously review the scientific literature and will adopt our research plans accordingly.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These 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 experience from our own studies on vaccination of tumour implanted mice about the expected data variation and group sizes necessary that will provide sufficient statistical power.
Individual experiments comparing two parameters would need 30 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experiments are based on our earlier studies. Therefore, we have extensive experience on techniques of studying tumour growth and experimental data variation. Future experiments will be planned and performed with advice from our collaborators, who have long-term experience in studying tumour growth and vaccination. This will help establishing optimal tumour implantation techniques to reduce experimental variation.

We will reference online tools NC3R's Experimental Design Assistant for experiment design, statistical analysis to predict the numbers of animals used in the study.

For each experiment, we will produce a written protocol. In this the numbers of mice used and the procedure they will undergo will be details, according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will apply good colony management to ensure the desired numbers and desired genotypes are generated with minimal wastage. Computer modelling may be used to predict experimental conditions that will show the largest effect sizes. At the end of the experiment we will harvest the maximal possible number of tissues. Tissues not immediately analysed will be archived frozen and will be made available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in all experiments.

Tumour models will be used to study B lymphocyte infiltration in tumours and interaction with other cells.

Mouse tumour cells (e.g. Lewis Lung Carcinoma Cell) will be subcutaneously injected into one single flank site of wild type mice or B cell reporter mice. Comparing to tumours that develop in the organ from which they are derived, subcutaneous implantation of tumours is one of the most refined tumour models because it minimally invasive method to administer cells, and subcutaneous tumours can be more closely monitored, allowing for better humane endpoints, and less likely to impact on animal welfare than those growing in organs and potentially impacting upon organ function. This will be the model of choice to determine how vascular targeting by vaccination and immune responses affect primary tumour growth.

Vaccination will be used to induce anti-tumour immune responses and test interactions of the immune response with the tumour. Mild adjuvants have been selected for immunisation that avoid significant pain or distress to the animals.

Why can’t you use animals that are less sentient?

Mice are the most appropriate species, because they have a huge degree of homology with the human immune system, and have been used extensively in previous studies meaning there is a wealth of information and established techniques available to us (e.g. gene manipulated strains, antibody and gene expression detection reagents). Adult mice have to be used because only the adult immune system efficiently reacts to the self-antigens we are planning to target with our vaccination strategy. The experiments run over several days and weeks and therefore cannot be done under anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures that may cause acuter pain, e.g. implantation of tumour cells by subcutaneous injection, will be done under short term anaesthesia.

Treatment with vaccine (e.g. immunogens, adjuvant) or modulators. These may have various side effects. Immunogens used in this project generally have little adverse effects, however we will use previous experience and data in the scientific literature to inform our use. Mice will be monitored appropriately after injection.

Implantation of tumour cells under the skin could cause adverse effects such as significant weight loss, distress or pain. Appropriate analgesia will be provided after injection and otherwise when necessary to reduce pain. The animals will be closely monitored after tumour implantation and humanely killed if any adverse effects are observed which exceed moderate severity. The subcutaneous tumour models is well established at our facility, and staff are expert at monitoring animals during the procedure and minimising adverse effect.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Experimental design, execution and publication will be done in line with the PREPARE and ARRIVE guidelines and in accordance with published best practice for 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 will reference online tools NC3R's Experiment Design Assistant for experiment design, statistical analysis to predict the numbers of animals used in the study.

For each experiment, we will produce a written protocol. In this the numbers of mice used and the procedure they will undergo will be details, all study report written according to the ARRIVE guidelines.

We will keep up to date following NC3Rs news and courses, attending NC3Rs and other 3R focussed events regularly offered at our institution. We have also signed up for the NC3Rs newsletter.

95. Studies of brain development in the mouse

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, development, neural circuits, neuro-developmental disorders, visual 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?

We would like to identify and understand molecules and mechanisms that ensure that neurons form the correct connections and neural circuits during development of the brain.

Our focus will be on neural circuits in the visual system and those controlling social behaviour.

Using similar techniques, we would like to identify those molecules and mechanisms which - if mutated or disrupted - are involved in the establishment of neural connectivity between the eye and the brain or/and in the aetiology of neurodevelopmental disorders such as autism spectrum disorders (ASD).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

For an understanding of the function of the brain, it is important to understand how different groups of neurons are connected to each other and how this is controlled during neural development. The precise connections between different groups of neurons are the basis of the function of the brain. However, there are thousands of different connections between thousands of different groups of neurons. To understand general principles of the set-up of these connections, researchers have to investigate a number of different connections and try to find common building blocks.

We would like to study a few of them. We are investigating first the connection between the eye and the brain which is already quite good understood. Here some molecules and mechanisms contributing to development of this neuronal connections have been already identified. We would like to understand these connections in further detail. We think this will help to better understand other connections and with this the overall function of the brain.

In contrast to this, an understanding of neural connectivity involved in neuro-developmental illnesses is still at its infancy. However, a few molecules and some neural connections involved in these illnesses have been identified and serve as a starting point for further investigations. The study of mice with genetic alterations in candidate genes for neurodevelopmental illnesses will provide important insights for a better understanding of these disorders.

What outputs do you think you will see at the end of this project?

We hope to contribute to a better understanding of the etiology of neuro-developmental disorders with particular emphasis on autism spectrum disorders. Our focus for the next few years will be an understanding of the role of microglia and astrocytes in this disorder, but we will be very open to further developments, either due to our own research, or through the publication by other labs.

Our output will be primarily publications in, if possible, general and high ranking journals. We will also communicate our findings via our webpages, and by attending scientific conferences, where we will present our data in form of presentations and posters, while also extensively networking with our colleagues.

Who or what will benefit from these outputs, and how?

Some considerable knowledge has been accumulated on the role of non-neuronal cell types in the brain, in particular microglia and astrocytes. Most of the functions of these glia are in supporting the function of neurons, however, there is also an extensive communication between these three cell types indicating that an understanding of the function of the brain requires an understanding of each of these cell types and their communication.

However, their role during the development of the nervous system, and in particular their role during the development of disorders of the nervous including autism spectrum disorders is much less understood. We would like to better understand the role of these glial cell types and their communication with neurons during normal development of neural connectivity and in situations where these functions are disturbed.

An understanding of the precise involvement of microglia and astrocytes in normal development is at the core of the research activities of numerous labs all over the world.

Some research findings indicate that if the function of these glia is disturbed, it leads to neurodevelopmental disorders. Therefore, an understanding of the processes which go wrong during neural development leading, for example, to autism, might be very helpful to find cures for bettering the conditions of those individuals affected by these disorders. Generally, it appears that in some cases of autism, more fundamental processes are disrupted leading to more severe cases, while in other cases the deficiencies are smaller and the individuals are less affected in their normal life. Therefore, autism is also described as a spectrum disorder.

Our projects will be of interest for these labs, since we aim to understand on a deep mechanistic level the role of microglia and astrocytes during development of neural circuits. New principles, concepts and molecules linked to these important cell types might be discovered in our projects. We think that the outcomes from our proposal might give new views of these cell types in the adult nervous system and might further an understanding of autism spectrum disorders during development, maintenance, stabilisation, homeostasis and repair of existing, adult neural circuits.

Furthermore, our research will be of intriguing interest for research labs working on the normal development of neural circuits, providing new starting points to understand these complex processes. One process we are in particular interested in is the control of the local connection points between neurons, the synapses, and we would like to understand here the pruning or removal of synapses in a process called autophagy or self-eating. Our pilot data might open-up a new area of research towards defining the importance of autophagy for the normal, undisturbed development of the brain.

How will you look to maximise the outputs of this work?

Naturally, we aim to publish our data in more general journals to reach a wide audience of neuroscientists, and aim to publish our research on web pages of the Institute, as well as communicate via social media including twitter.

We will communicate our results on meetings and congresses which have a wider spread of covered topics including neuro-developmental disorders. We aim to attend meetings which have a focus on autism.

Species and numbers of animals expected to be used

- Mice: 2000 wild type mice; 2000 transgenic 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.

We will use mice since for this organism relevant, well-established models are established to study on a genetic level neuro-developmental disorders such as autism spectrum disorders (ASD). These models comprise (conditional) knock-outs and knock-ins of candidate autism genes. We will be working in particular with genes, for which it has been shown that in humans mutations in these genes are associated with neuro-developmental disorders.

Since ASD is a disorder affecting early neural development, we would like to analyse in particular early, postnatal, states of neural development in these model systems.

Additionally, we would like to extend our studies on the development of topographic projections, in particular the retino-collicular projection (I.e., the connection between the eye and the brain).
Topographic projections describe the axonal connections between groups of neurons in which neurons which are neighbouring in one area are connected to neurons which are neighbouring in the other area. In this way positionally organized information can be communicated between different groups of neurons. We will use the mouse system since only here particular mouse lines are available, in which select key molecules can be removed or inactivated very locally and in a very time dependent manner.

We will study early postnatal stages.

Typically, what will be done to an animal used in your project?

One major strand of research in my lab will be the investigation of neural circuits in the cortex, which are affected in neuro-developmental disorders (for example the medial prefrontal cortex). We will be analysing here typically the morphology, function and connectivity of neurons, microglia and astrocytes in mouse lines with genetic alterations in candidate autism genes and compare them to the wild type situation.

Another major strand will be a functional approach, in which we will alter the function of microglia and of astrocytes during early postnatal development, and study the consequences of these interventions on the morphology of neural circuits in the cortex of wild-type and mutant mice. We also will study consequences of these interventions on the behaviour of these mice in typical ASD behavioural assays.

For analysis of topographic projections, we will fluorescently label local areas in the retina using fine needles and analyse a few days later the connections of these retinal cells within the brain.

What are the expected impacts and/or adverse effects for the animals during your project?

In structural investigations of neural circuits in the cortex we will use a variety of mouse mutant lines. For these, we do not expect adverse effects. At the moment, it is not planned to generate new mouse lines. The existing mouse lines we would like to use are very well characterized, and their characterisation so far does not suggest increased pain, weight loss and/or tumors. Abnormal behaviour in terms of social interactions, repetitive behaviour, ultrasonications and a tendency for increases in epileptic seizures have been reported.

In our functional approaches, we will interfere with the function of cells in the CNS, with particular emphasis on microglia and astrocytes, and study their consequences. These interventions have been principally already performed by other labs, and no gross phenotypes in terms of pain etc. have been reported.

The combination of approaches we are planning in terms of mutant mouse lines, functional interferences, and behavioural studies, have not been carried out so far.

Expected severity categories and the 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 functional characterisations will involve the administration of tiny amounts of substances to the brain and to the body of young mice (wild type and mutant). These approaches involve small operations, meaning that the expected severity of the approaches will be medium.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We would like to better understand the aetiology of autism spectrum disorders. These disorders are believed to be caused predominantly by genetic mutations in genes which control neural circuits and eventually behaviour.

For this, we need to work with intact mouse models, since the read-out of ASD will be a behavioural analysis which is controlled by the complex interaction of multiple groups of neurons. We therefore have to interfere on a molecular level with the function of neurons, microglia and astrocytes in vivo, and study the consequences of these interventions on their behaviour.

Which non-animal alternatives did you consider for use in this project?

We have considered to study, for example, the interaction between neurons, microglia and astrocytes *in vitro*. These approaches will likely generate further knowledge on molecules and mechanisms involved in these interactions.

Furthermore, brain development in mammals involves a complex choreography of events that are poorly understood. For this reason, it is currently inconceivable that we could generate computer models to advance our understanding of brain development and the disorders that affect this process.

I have checked at the frame web site (www.frame.org.uk) and there are no current alternatives to my research on brain development on animals.

Why were they not suitable?

However, these results have to be taken with great caution, since there are time- and region-specific interactions in the brain between these cell types, which only exist *in vivo*, which will be disrupted if one or the other cell type is put in culture, and where they can be

functionally and structurally investigated.

A well documented example are microglia, which when taken out of the brain context, rather dramatically change their functional characteristics in culture.

Nevertheless, we will use organotypic cultures of brain slices whenever possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have from the beginning of our experiments, a clearly outlined set of breeding based on year-long experience to achieve our experimental goals.

We do know which mouse lines we would like to analyse; we do know the number of mice for each parameter to be investigated, we do know the number of breeding to achieve this number of mice. We have a clear idea which ages we would like to investigate. We have a clear knowledge which controls to include. We have a clear knowledge to plan for back-up breeding in case some experiments go wrong.

Our own year-long experience is substantiated by our knowledge of approaches of other labs to achieve functionally and statistically relevant data, based on their publications.

We will constantly aim to improve our statistical methods. We will also seek for statistical advice in very complex experiments. Based on previous experiments, with a power of 80% and an effect size of 20%, our sample size varies from 4-5 animals (e.g., quantification of the number of cells) to 12-15 (e.g., behavioural tests).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have more than 20 years of experience using mice as a model system for brain research. My research project is designed to minimize the number of animals used to obtain statistically significant results when testing our hypotheses, including the use of factorial experimental designs.

We will review our mouse colony on a weekly basis, and will reduce the number of mice if possible.

We will investigate ways to analyse experiments in multiple ways so that with fewer mice more data become available.

Because the mouse brain is relatively large, for structural investigations we will generate hundreds of sections from one brain, allowing us to obtain material for multiple sets of experimental approaches.

In addition, we use a highly developed database that aids us in obtaining the precise number of transgenic mice required for our experiments. Furthermore, we collect the brains of all the mice that we used in our experiments (including many from mice that are only used as breeders) and we store all the relevant information in a database.

In addition, we make this material available to other researchers in our Centre, which also contributes to reducing the number of mice used in experimental procedures.

Personnel in my laboratory will take the online course designed by M. Festing (www.3rs-reduction.co.uk) to minimize the number of animals before starting their experiments.

Only a fraction of the total number of animals that will be breed will be used in experiments. This is due to the fact that the desired genotypes (e.g. a conditional mutation) are generated along with non-desired genotypes. For example, breeding might involve crossing of mice with floxed alleles and the cre -driver allele, however, in the offspring a few pups would not have the desired phenotype (homozygous floxed, cre positive). Those mice, for example pups without the cre allele, might not be of relevance to be included in the experiments and therefore killed using a Schedule 1 procedure.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Apart from good experimental design, we will review the number of mice and the ongoing breeding on a weekly basis.

We will review our results on a routine basis, and change our experimental design accordingly.

We will aim to record as much data as possible from each experiment so that experiments do not need to be repeated as often.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the mouse as the animal model system. Methods used are structural and functional investigations of most suited (conditional) knockout mice of candidate ASD genes. The mouse model system is the only model system with such a variety of genetic tools and already available mouse lines to study ASD. For this purpose, the mouse is the best possible animal model because their genome is relatively easy to manipulate and their brain develops in a similar manner to humans. The use of conditional mouse mutants, in which only a specific population of neurons lacks the gene of interest, provides the most accurate results and hence refinement. In contrast, full knockouts in mouse – but which are available also in other species -often have disadvantages since here the protein/gene

of interest is removed from the entire animal which could have multiple effects, and which might be difficult to understand.

Why can’t you use animals that are less sentient?

To study the etiology of ASD in the context of neural circuit development linked to behaviour, we would like to use a model system as close to humans as possible. Therefore, we are interested in using the mouse model system with its huge genetic resources, while other model systems such as zebrafish and Drosophila appear much less suited.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will constantly aim to improve our statistical methods.

We will investigate ways to analyse experiments in multiple ways so that with fewer mice more data become available.

In particular, for structural investigations we will generate hundreds of sections from one brain, allowing us to obtain material for multiple sets of experimental approaches.

Finally, 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 sacrifice for the experimental procedures, using approved methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will get our information on how to conduct experiments in the most refined way from the relevant NC3R web pages. We also will be in close contact to the local Ethical Review Committee.

Personnel in my laboratory will take the online course designed by M. Festing (www.3rs-reduction.co.uk) to minimise the number of animals before starting their experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will rountinely on a monthly basis visit relevant web pages to look for updates, such as the ones from the ' UK National Centre for the replacement, refinement and reduction in animals in research NC3Rs), and the web page ' understanding animal research ' .

Recommendations will be implemented imminently.

96. Understanding cortical plasticity and learning

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Memory, Learning, Neurone, somatosensory, cerebral cortex

Animal types	Life stages
Mice	juvenile, adult, pregnant, embryo, neonate, 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 understanding learning and memory processes in the cerebral cortex and the synaptic plasticity mechanisms that underlie them. By understanding synaptic plasticity in healthy brains, we will gain insights that will help us design treatments for psychiatric and neurological conditions in which plasticity is impaired such as schizophrenia and 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?

It is important to undertake this work because the synaptic processes that underlie learning and memory are defective in several conditions that afflict people and so far, therapies are

lacking. Synaptic plasticity conditions can broadly be divided into two main categories, (a) neurodevelopmental conditions, where defective plasticity during development leads to miswiring of the nervous system (examples here would include schizophrenia, autism, and fragile X), and (b) neurodegenerative conditions where the neuronal circuit underlying learning and memory is initially healthy but degenerates with time and can no longer create or recall memories (for example Alzheimer's disease). By understanding how synapses are modified, which neuronal circuits control synapse modification and how these processes are affected by abnormal development we may be able to develop therapies to treat these conditions.

What outputs do you think you will see at the end of this project?

Our main outputs from this project will be publications in scientific journals. We aim to produce information on how neuronal circuits control changes in synaptic structure that lead to enduring memories. We aim to produce information on how information is transmitted from sensory parts of the cerebral cortex to the hippocampus where short term memories are stored and how the short term memories become consolidated into long term memories during sleep. We aim to produce information on how neuronal circuits can go wrong due to defective development in neuropsychiatric conditions such as schizophrenia. Finally, we aim to understand the potential benefit of nascent therapies for restoring synaptic plasticity in conditions in which it is lost such as Alzheimer's disease.

Who or what will benefit from these outputs, and how?

Short term Impact:

The short term impact of our studies will be realised within a year or so of the start of this licence and will mainly affect scientists working in the area of neuroscience who will read the papers we begin to publish. They will benefit by gaining greater understanding of how cortical plasticity works and what factors within the neuronal circuit affect synaptic changes underlying memory formation. The fields of academic research that will be affected are expected to be those of Perceptual learning, Psychiatric disorders, Cortical processing of information, Sleep research, Somatosensory processing. As the primary research articles are published, we will integrate this information with that from other labs to write review articles and hence disseminate the knowledge to a wider audience, including academics, medics and researchers in industry.

Longer-term Impact:

The longer term impact of our research will be to create a more complete understanding of how synaptic plasticity occurs from the level of the molecular mechanisms at the synapse, to the level of the neuronal circuit in the cortex, where several elements dynamically interacting to generate synaptic changes under some conditions and not others. This will create a test-bed system that can be built on to
(a) further understand the mechanisms involved and (b) to understand how these synaptic and anatomical pathway mechanisms are disrupted in psychiatric and neurological conditions and (c) to test therapies for improving synaptic plasticity and hence memory function.

How will you look to maximise the outputs of this work?

We will collaborate directly with academics at our home establishment with whom we

jointly hold grants for research in the departments of Bioscience and Psychology. These collaborations will create synergies between our work on somatosensory cortex and touch memory and the work of scientists currently studying plasticity in the visual and limbic systems. We will therefore create a more generalised and holistic understanding of learning and memory by combining understanding with our studies. Our interactions are likely to influence one another's studies within our own subfields and hence help us to disseminate knowledge further afield via non-overlapping components of each others' professional networks. We will also interact with the wider home institute's neuroscience community, including those located in medical centres aiming to understand human psychiatric conditions through genetics. Interactions will naturally occur via seminars, symposia and workshops. Our work can help them to explain the functional consequences of the mutations and molecular pathways they identify in the human population. Internationally we will interact with neuroscientists in Japan and South Korea via annual symposia sponsored by the Medical Research Council and with neuroscientists from all over the world, who meet at the annual Society for Neuroscience meeting in the USA.

In order to maximise the impact of our findings, we will publish our studies in prominent internationally read journals and publish reviews and public engagement articles for a wider audience. An integral part of our publications will be to describe what did work and what did not work in the experiments (or trial experimental therapies).

Species and numbers of animals expected to be used

- Mice: 2000
- 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 wish to use rodents in this study because they are the lowest sentient mammals that have a similar cortex to humans. Because they are relevant to humans, rodent studies allow us to probe the effect of genetic mutations present in the human population that lead to psychiatric and neurological disorders. Mice are a commonly studied laboratory animal and are available in many genetically mutated forms. We need to use the mouse genetic mutations available in the research community for two main reasons
(a) because they mimic human mutations that predispose people to psychiatric and neurological disorders; we can gain insight into the causes of these conditions by studying the mouse genetic models. (b) several strains of mice allow cell-specific expression of artificial ion channels that can be stimulated optically and chemically in order to probe particular anatomical pathways. In addition, because our lab has specialised in plasticity in the barrel cortex and visual cortex of the mouse, we have extensive prior experience and data that can be used to predict sample size and understand the new data as it is generated. In some circumstances, rats are a preferable model to study. This is usually because they are larger, which allows them to carry head implants more easily and occasionally because their behaviour is more accurately measured and interpreted.

Typically, what will be done to an animal used in your project?

Animals used in these studies will typically carry prior mutations that enable them to express genetic material in a cell specific manner or to mimic a particular psychiatric or neurological condition. We will need to breed animals of known genotypes to do this. Consequently, we will need to take a small ear sample biopsy to run a PCR based genotyping typically at the stage when the animals are weaned.

The following procedures will be use to understand synaptic plasticity

Animals will receive an injection of virus to transport the genetic material to the neurones under investigation so that we can see the neurones and manipulate them.

Some animals will receive a cranial window implant during the same surgery that delivers the virus. This enables us to see and manipulate the neurones under study over a period of several weeks.

The animals will then be trained on behavioural tasks where their learning is assessed under various conditions, which typically comprise optically activating or inactivating subsets of neurones .

Some animals will learn a tactile texture discrimination in a free-moving task in order to obtain a food reward. The mice will have food restriction during this period.

Some animals will learn a very similar texture discrimination while located under a microscope in a head-fixed apparatus to which they will have become acclimatised over several days. They are able to move while in the head-fixed position by walking or running on a ball or track. They will receive a water reward during this period having been on water restriction before hand.

Another group of animals will receive the viral injections but then be killed rapidly by decapitation and brain slices prepared for detailed intracellular micro-electrode recordings in vitro.

Rats will receive an electrode implant and following a recovery period of several weeks learn the same free-moving texture discrimination for a food reward. The animals will have undergone food restriction during this period.

Some animals will have received viral injections as described above but then be anaesthetised and positioned in a stereotaxic frame and the cortex exposed for electrode recording while stimulating sensory inputs. These studies will be concluded under the same anaesthesia without recovery.

Some animals may undergo sensory deprivation, which either involves suturing closed one eyelid, being placed in the dark for a few days, or having their whiskers trimmed. Following these procedures the animals will either be placed in stereotaxic frame and electrodes used to record sensory responses or be rapidly decapitated in order to prepare brain slices.

What are the expected impacts and/or adverse effects for the animals during your project?

For some animals the impact is mild or negligible as anaesthesia is rapidly induced with isoflurane before the animal receives an injectable anaesthetic from which they do not

recover. For the animals receiving head implants, viral injections and electrode implants, the adverse effects is a week or so of recovery from the surgery, which is mitigated by analgesics. For the animals undergoing behavioural studies, those undergoing food restriction will lose some weight compared to when fed ad libitum, but we will monitor this closely and ensure it is not more than 15% of their initial weight. The animals undergoing the head-fixed behavioural task will endure some anxiety until they learn that the apparatus is safe and they are used to it. These animals will also undergo water restriction and so may feel thirst, which can be sated during the experimental learning task. The implants are typically in place for 3 weeks before experimental handling and acclimatisation to the apparatus begins. Behavioural studies typically carry on for a further period of 3 weeks beyond that.

Expected severity categories and the 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:

Breeding only (20%) subthreshold

Viral injections followed by non-recovery anaesthetic and stereotaxic recording or preparation of brain slices (30%) Moderate

Viral injections followed by head-fixed behavioural tasks (25%) Moderate

Viral Injections followed by free-moving behaviour with or without intermittent imaging under anaesthesia (20%) Moderate

Rats:

Viral injections and electrode implantation followed by free moving behavioural studies (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?

Animals have to be used because the subject of the study is how learning affects the plasticity of synaptic circuits in the brain. Therefore, we need a whole animal with an intact brain and all its motor and sensory apparatus available to acquire information from the animals environment.

Which non-animal alternatives did you consider for use in this project?

Cell culture Computer models

Why were they not suitable?

Cells in culture are immature and so cannot mimic the plasticity present in the adult brain. Cell cultures are not linked up to the sensory apparatus of a whole animal and are unable to learn about the environment. Finally, cell culture lack the complexity of the brain which is composed of billions of cells organised in several different functional subsystems.

At present we do not know enough about how plasticity works to model how experience affects the brain in a computer model. If we did know how to make such a computer model we would still need to check its validity using animal 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 have calculated the number of experiments we anticipate performing and the numbers of animals required for (a) breeding the experimental animals (b) determining whether the experimental results are valid and reproducible. We know from previous years' animal use that we are approximately correct in these calculations. We have also allowed a buffer in case some experiments fail for technical reasons or unforeseen difficulties.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used power analysis to estimate the number of animals necessary for statistical validity of our studies using an alpha value of 0.05 and power of 0.95. We used prior measures of effect size and variance for the power analysis, largely from our own previous data but occasionally using data from other labs. We multiplied the number of variables in the studies by the group size needed for statistical significance to arrive at the total number of animals that would typically be required. We took into account that animals in each comparison would be of similar age and size and balanced for gender. We took into account that in some cases we could use each animal as its own control, while in other cases we needed to compare across cohorts of animals (for example different genotypes or 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?

Where we have not already done so, we will undertake pilot experiments to improve technical measurements and optimise animal learning procedures and apparatus.

We will use efficient breeding to keep the number of experimental animals generated to low levels. In several of the genotypes that we plan to use the heterozygotes exhibit the

phenotype. Where double heterozygotes are required we can use the single heterozygote progeny for further breeding or often as wild-type controls.

In some experiments where brain slices are produced, we may be able to share the tissue between two researchers on different recording rigs in the same lab at the same time to maximise the number of cells recorded from each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rodents in these studies, mainly mice and a smaller number of rats. We will use the somatosensory cortex as a model system for how different cortical areas interact to create the conditions for plasticity during learning. We will minimise suffering in every case by the use of anaesthetics and analgesics. We will minimise the chance of animals suffering from infection by using aseptic techniques for surgery. We will minimise the chances of animals with head-plates becoming entangled in their home cages by housing them in large cages. We will minimise the chances of the head-plates becoming detached by using a combination of strong adhesives. Animals undergoing food or water restriction during their learning tasks will be carefully weighed regularly (usually every day), and their health closely monitored.

Why can’t you use animals that are less sentient?

Examples of less sentient animals might include flies, fish and worms. While these animals can learn, they do not have the same brain structure as rodents, which do have a cerebral cortex that is similar in form to human cortex. Given that we would like to understand how the human brain learns and what goes wrong in the plasticity processes underlying psychiatric and neurological conditions, we need to use a species with a similar enough brain to make the studies relevant.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals are monitored almost continuously during the recovery phase from surgery to ensure they are feeding, drinking and moving well. Pain management is controlled by prophylactic analgesics before surgery and afterwards in the drinking water as needed. All surgery is conducted aseptically, topical antibiotics are used and all wound margins sealed to prevent infection routes. Animals are left to recover for several weeks before introducing them to the behavioural setting. Animals undergo several weeks of gentle handing and general acclimatisation to the apparatus before we begin training. We will explore new published methods of delivering Clozapine N-Oxide (CNO) via a micropippette as an alternative to intraperitoneal injection of the drug.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will broadly follow the methods outlined in the Animal Research Reporting in vivo Experimentation (Arrive) guidelines in order to use animals in the most efficient way. These guidelines describe high quality methods and standards for reporting the results of in vivo animal research and are recognised internationally. They cover the content required for each section of a paper from the Title and Abstract through to the Discussion. The recommendation on the Methods and Results sections are particularly important and include detail on clearly stating many aspects of the study from study design, experimental procedures, housing and husbandry and statistical measures through to outcomes and estimation of precision of measurements. An updated version has been published in PLOS in 2020 (https://doi.org/10.1371/journal.pbio.3000410).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are constantly in touch with the animal care staff at our home institute and receive regular bulletins from NC3Rs about advances. We frequently attend international meeting where best practice is discussed. We have a Regional Programme Manager from the NC3Rs working with our establishment and benefit from advice and updates.

97. Genetic modification of chickens to understand vertebrate 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

embryonic development, organogenesis, tissue fusion, organ size, developmental timing

Animal types	Life stages
Chickens (Gallus gallus)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to modify specific genes in chickens to understand their function during vertebrate embryonic development. The results from these studies will improve understanding of the genetic regulation of normal vertebrate development and the causes of human and animal developmental 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 chicken, a non-mammalian animal, is a valuable and tractable model for understanding the genetics and developmental processes important to form a healthy embryo. Understanding the genes that control development and growth of the chick embryo and how these regulate the inherent cell behaviours and molecular interactions in these processes, is important to help elucidate the causes of human developmental defects and how these can be treated or cured, but also will inform the study of developmental processes common to other vertebrates.

There are many human developmental defects, and a large proportion of these are genetic in origin. One example of these is tissue fusion disorders, such as cleft palate or spina bifida, or coloboma of the eye. The incidence of these can be as common as 1 in 500 new births. Many of these defects cause significant morbidity throughout the life course and currently have no cure. In addition, multiple birth defects do not have their causative gene (or genes) yet identified, reflecting a gap in our knowledge of the relationship between genes and the processes that they regulate. One major way we can acquire knowledge of gene function in development is through studying normal and abnormal developmental processes in vertebrate embryos, with particular emphasis on how these genes are turned on and to what levels, when and in what tissues. These are major outstanding questions in our understanding of normal embryonic development and the causes of human birth defects. Such studies can also lead to preventative measures being taken to reduce the number of birth defects, a good example for this is taking folic acid during pregnancy.

The value of the chick embryo as a model for vertebrate development continues, with added value conferred by access to transformative improvements in genetic modification capabilities; these have been pioneered and are readily available at the host Institute. The chick embryo can be easily accessed in the egg and manipulated experimentally (without affecting the mother as is the case in the mouse). In addition, many of the key developmental processes take place before the regulated stage of embryonic chick development. Indeed, for this licence, the majority of work will be performed in embryos prior to embryonic day 14. In parallel with the development of genetic modification technologies for the chicken has also been the freely accessible publication of the complete chicken genome. This widens the scope, utility and validation potential of genetic modification approaches and functional genomics in the chicken. It is important to now

utilise these advantages of chick experimentation to address the needs of developmental biologists and human geneticists and clinicians in understanding the causes of developmental birth defects, and the processes that underlie normal development.

What outputs do you think you will see at the end of this project?

The intention is to combine the generation of new genetically modified lines with complementary transient transgenic approaches where possible. It is expected that up to 10 new genetically modified lines could be generated throughout the duration of this project, and in all of these the majority of work will be performed in unhatched embryos. The work will help identify the genes that are important for the development of healthy chicken embryos that will be valuable for improved understanding of human embryonic development. We will also gain more understanding of how certain faulty genes can lead to birth defects affecting the face, eyes, and limbs, and other tissues or organs. We will be able to make progress in determining what cell behaviours these genes control during development, and how these processes are coordinated to produce specific organs or tissues from small numbers of starting cells.

This project will also develop improved methods and approaches to generate more data from each single embryo by selectively isolating the specific cells of interest for analysis of genes or proteins. This will reduce the number of animals used for this type of research. During this study we will continue to make available these novel tools to other academic researchers and to develop additional genetically modified lines that will significantly enhance the knowledge gained from studying vertebrate in the chick embryo.

Our results will be published in scientific journals and communicated to developmental biology and clinical genetics research laboratories worldwide.

Who or what will benefit from these outputs, and how?

The work will improve the knowledge base among biologists. For example, those in the fields of eye development, tissue fusion, limb development and craniofacial biology. Many findings will have broader relevance to biologists working in other developmental contexts (e.g. palate, neural tube and heart development), or in disease contexts affecting patients over the wider life-course, e.g. protein interactions and cell behaviours in cancer or neurobiology studies, or offering an improved understanding of the molecular basis of ciliopathies. These beneficiaries will benefit directly through publication of new experimental evidence of the key cells, their shape changes and behaviours, and the precise mechanisms, genes and molecular interactions involved during these developmental and disease processes. This will improve understanding of which processes are essential for development, where and when in the embryo these are required, and how they are likely to cause disease if not correctly regulated at any stage of life.

The work described in this study may have a wide impact on developmental biologists currently working in other model species such as mouse and zebrafish. The chick embryo has traditionally been lauded for its utility but has been hampered by the lack of genetic modification methodologies. Work in this study will bring the tractability of the chick in line with these other species, with the underlying benefits of chicken embryology, such as the ability to synchronise development between eggs/embryos, and the ability to keep the mother alive to continually produce genetically modified offspring (in contrast to mouse). In general, developing tissues in the chick embryo are larger and provide more cells to

perform experimental analysis, and can be cultured outside the egg and used for live microscopy.

These advantages may provide the basis for other developmental biologists to select the chicken as their future model organism of choice, making a significant 3Rs improvement to the field of developmental biology research.

Clinical geneticists with interests in a range of birth defects, such as the causes structural eye defects, limb abnormalities and craniofacial disorders will also gain new knowledge into mechanisms of disease causality and the identification of novel candidate disease genes. Any new genes revealed from these studies will be shared to be included in patient screens to identify potentially causative mutations from large gene sequencing projects, leading to improved molecular diagnostics. Examples of this are the NTN1 and SMOC1 genes in eye patients, and the TALPID3 gene in ciliopathy patients. In the longer term, these studies will inform genetic counselling for families affected by developmentally regulated birth defects.

How will you look to maximise the outputs of this work?

We collaborate internationally and communicate our scientific results at seminars, conferences, 3R conferences, and through scientific publications and a combination of broad scope and specialist open access journals. We will endeavour to publish all results, whether successful or unsuccessful, in a timely manner in high-ranking open access journals with broad scope.

We communicate with other laboratories who are also developing avian genetic modification techniques for developmental biology, and who work on the same tissues or organs in other species. We will utilise well trained bioinformaticians to analyse the gene expression data.

In particular we will maintain and expand collaborations with clinical geneticists to maximise the impact of this work. These will be strategically developed with world-leading clinicians, e.g. with specialities in both ophthalmology or limb defects, and those with interests in broad syndromes. In particular we will seek those with large patient cohorts. These collaborations will allow us to expedite clinical relevance of findings, or to determine which aspects of our research to focus on for maximum clinical benefit.

Species and numbers of animals expected to be used

- Domestic fowl: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The chicken embryo is an appropriate and well established animal model to study vertebrate development and the basis of human developmental disorders. The chicken is very useful to study embryonic stages as a fertilised egg can be obtained without surgery on the mother. To develop genetically modified lines we need to use adult birds, and to

enable us to study gene function and cell behaviours in some contexts, we may need to use hatched or adult birds.

Typically, what will be done to an animal used in your project?

The animal will be blood sampled one to five times during its life. Its behaviour may be observed. It may be treated with a drug to change gene expression or to label or ablate cells in the animal. The animal will be humanely euthanised after which phenotypic and genotypic data will be obtained.

What are the expected impacts and/or adverse effects for the animals during your project?

There is a reduced hatching rate experienced for some of the lines of chicken and from manipulated eggs. Most of the chickens will lead a normal life. There is the consistent problem to breed homozygous animals for a genetic locus while avoiding the health deficits associated with inbreeding of chicken lines. We will maintain an up-to-date list of all genetically modified birds on a public website with details of linked publications and 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)?

Mild severity: 95% of animals Moderate severity 5% of animals

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The biology of embryonic development in birds is of interest in terms of understanding developmental mechanisms for all vertebrate animals (including humans or farmed animals), while the developmental process that occur in the embryo are also what determines good health throughout the life of the animal. We will keep the use of animals to a minimum by focusing work in this licence on embryonic stages prior to embryonic day 14.

The use of embryos and animals is necessary to study the function of genes during a range of developmental stages throughout the whole organism, and in physiologically-relevant contexts. This is particularly the case in genetically modified models where a gene or region of DNA sequence has been perturbed. Gene expression, specific cell behaviours and molecular interactions in an embryo are a result of each cell’s ability to interpret cues

from secreted growth factors, cell-cell signalling, and the interpretation of positional information within a developing tissue, in combination with the physical stresses on each cell. The responses and cellular interactions within live embryonic tissues are also highly dynamic through developmental time and the life of the animal, which allows complex structures to develop and be healthily maintained, such as in the eye or limb. The combination of all of these parameters are what determines the phenotypic outcome, and this cannot currently be replicated in existing cell culture systems, or explant or organoid cultures. For example, the organoid culture system developed to understand early eye development does not undergo the complex process of tissue fusion or the structural development of many other ocular structures. Similarly, existing limb cultures do not readily recapitulate the early development of limbs and the complex arrangements of bones, tendons and musculature. Therefore, to determine the precise role for a gene during the complex development of an organ, it is vital that the organism is intact when such developmental studies are carried out.

The use of the chicken as a model species itself addresses “replacement” to the extent that licensed experiments carried out in mice may be replaced by the use of genetic modification chicken embryos from lines created under this licence, for studies before the regulated, late stage of development.

Which non-animal alternatives did you consider for use in this project?

In vitro organoid culture systems have been developed to generate embryonic eye tissues from stem cells and these were considered for aspects of this study.

We will use cell cultures to provide supportive data for some experiments. For example, to identify the interaction partners of some encoded genes or to explore upstream influences on the expression of specific genes.

Why were they not suitable?

The in vitro organoid culture system to generate eye tissue does not produce the complex folding and tissue fusion events seen in the developing chick eye. Organs such as the limb, which consist of populations of cells from multiple embryonic origins, have not been cultured in vitro through all developmental stages. Therefore, this approach is not suitable for the eye development work in this project.

Investigation of some aspects of development will be carried out using in vitro systems where possible, but comprehensive studies, for example the impact to a particular region of the embryo of the loss of a specific gene, the fate of cells in a developing tissue, or cell behaviours in a complex 3D tissue framework, are only possible in a whole animal. Indeed, the study of eye, face, skeleton or limb development are not possible in other non-vertebrate models such as flies and worms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 total number of genome edited and transgenic lines we will establish over 5 years, and the number of genome edited offspring that will be bred during that time period. For each genome edited line, we will need to generate surrogate host birds that will carry the genetic material and use wild type birds for experimental crosses and breeding to maintain these lines. For each genome edited line, tissue-grafts or other experimental approaches, we will use power calculations to determine the number of animals we need to breed or use in order to carry out the required experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The cell culture of reproductive chicken cells enables us to carry out some experiments without using animals. The donor reproductive cell injections are carried out at unregulated developmental stages in eggs and many of the experiments will also be performed before the chick would hatch. The use of avian species as models offers advantages over mammalian species for the study of embryonic development. In this instance, the chicken offers advantages over using a mouse models as the mother does not need to be culled to obtain the embryo and a surgical manipulation is not needed to introduce donor reproductive cells into the embryo.

The majority of experiments will also be carried out at unregulated developmental stages (before day 14 of incubation) in the egg. Most aspects of eye, limb and facial development have occurred in the chicken embryo before day 14 of incubation so early development can be studied in embryos before they would hatch.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Experimental designs will be developed with advice from our Institute statistician who is a member of our AWERB. The number of birds required to maintain breeding lines of genetically modified birds will be kept to a minimum (2-3 pens) required from our past experience of the numbers required to ensure continuity of a line. Birds of a specific genetically modified line will be bred at greater numbers where specific project requirements need greater numbers of birds for limited periods. Discussions with colleagues and wider collaborators developing and utilising genetically modified mice or other available species will also ensure we benefit from advances in emerging genetic modification processes to ensure the number of animals used are continually kept to the minimum required. Flocks are maintained in known families at a size which balances adequate material balanced with maintaining a healthy in-breeding co-efficient (approximately 16 females 2 males per family).

Where possible, we will always endeavour to utilise multiple tissues from each embryo or sample to maximise the experimental outputs from individual samples. Similarly, we will make use of Institute freezer facilities and standard tissue fixing protocols to store samples for future work. All samples will be considered precious.

We have worked to improve the efficiency of hatching surrogate chicks carrying donor genetically modified reproductive cells. We will make use of genetically modified surrogate hosts birds (male and female) that lay 100% of the offspring from the donor genetically modified cells. We have improved our genetic modification of the donor genetic material

(chicken cells in culture) to screen for the genetic modification before the genetic material is introduced into the surrogate host chick embryos. We are also developing methods to reduce breeding by directly mating the surrogate hosts to generate pure offspring in a single generation which greatly reduces the number of chickens bred in these experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The chicken is both a farmed animal of major importance to human nutrition worldwide, and a versatile model organism for the study of vertebrate development. Increasing basic knowledge of the physiology and development of the chicken that can only be investigated in the intact embryo/bird, and that involves understanding tissues and organs in the intact animal, is the focus of this PPL. Applications of genetic modification technologies in biotechnology and genetics also must relate to the function of the intact animal. The studies are designed to be as minimally invasive as possible; the vast majority of birds will only be blood sampled to confirm genetic modification or transgenesis and then used for breeding as required.

All new transgenic and genetically modified lines are planned to target genes where there are no expected adverse phenotypes in heterozygous birds, which will be used for crosses to generate embryos for experimental analyses. These heterozygous genetically modified birds will be closely monitored for any adverse phenotypes. Furthermore, within this licence the significant majority of work will be performed in chicken embryos prior to embryonic day 14 when they become recognised as protected animals under current UK legislation.

The use of cultured avian reproductive cells that can be genetically modified to disrupt gene function and used to produce genome modified birds is unique to the chicken and avian species. The access of the fertilised avian egg also means the surrogate host chicken can be hatched without killing the mother to obtain the egg. The transfer of reproductive donor cells into surrogate hosts is done in the laid chicken egg at young ages (unregulated stages) before the formation of a nervous system. The surrogate hosts chickens will grow normally but make eggs and sperm from another chicken breed or potentially another bird species.

Why can’t you use animals that are less sentient?

The majority of work in this study will be performed on chicken embryos. There are few vertebrates that are experimentally tractable as chick embryos, or that mimic the developmental processes and genetic regulation of humans as closely. In addition, chick embryos undergo many of their organogenesis and structural organ or tissue development processes prior to day 14 (2/3) of gestation. The chicken offers further advantages over

using mouse or other rodent models as the mother does not need to be culled to obtain the embryo, while a surgical manipulation is not needed to introduce donor reproductive cells into the embryo.

The study of eye, face, skeleton or limb development is not possible in other non-vertebrate models such as flies and worms as the organs and tissues of these species lack the complexity of higher vertebrates. This is also largely true for fishes as they are cartilaginous and also do not have closely comparable limbs to mammals. Although some fish eye development processes have developmental similarities with humans, the chicken is structurally more similar and has more available tissues from which a larger number of cells for experimentation can be collected and analysed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have selected genes for modification where heterozygous animals are not predicted to be adversely affected. For all new lines, we will instigate increased monitoring for the weight and behaviour (feeding, social) of newly hatched novel genetically modified chicken lines to verify wellbeing and reduce suffering for all hatchlings. For new genetically modified chicken lines all preliminary work will be performed in embryos to assess any deleterious phenotypes. These will primarily be aimed at early embryos during the first 1/3 of development, then at ½ gestational stages, before assessing phenotypes at pre-hatch late stages in the final 1/3 of gestation. This strategy will be employed to specifically identify any potential welfare costs to subsequently hatched birds.

For any new lines that show an unexpected adverse phenotype we will immediately consult our Named Veterinary Surgeon to assess the welfare issues and to discuss pain management or therapeutic strategies. We will also aim to reduce the number of animals hatched and maintained with the adverse phenotype, and will consult with the Home Office Inspector to agree on best practice in this scenario and conduct additional harm-benefit analyses.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All work is undertaken in close collaboration with our Named Veterinary Surgeons. Study protocol forms must be completed and submitted to them prior to proceeding with any experimental work and animal unit staff are closely involved in experimental work and will offer advice on improvements to experimental protocols and monitoring/raising of the animals.

The group contains all the competencies required to perform the majority of techniques and procedures outlined in this project. Where additional expertise is required, staff will undergo appropriate training until competent.

Animals will be kept in accordance with annex III of Directive 2010/63 EU and/ or Home Office Codes of Practice, as appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We communicate with our NVS on current developments to enrich the environment of

housed chicken. Our poultry workers are trained in proper welfare techniques for birds and we are strictly monitored by our veterinary scientists and the Home Office on current practices. We visit the NC3R website for new information on animal use in research, and are kept up to date with associated scientific literature. As part of this we will access and complete relevant e-learning resources that are freely available for training and continuing professional development on the NC3Rs website. We will also remain in close contact with veterinary staff and attend annual conferences covering veterinary ethics and welfare.

98. Translational Cancer Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immunotherapy, Liver

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 direct anti-cancer and immune-mediated therapy for various cancers, including primary liver 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?

There are several potential benefits likely to derive from this project including:-

Development of more informative tumour models

Optimisation of single-therapy, combinations and schedules of therapy

Provide rationale for early-phase clinical trials

We anticipate that our experiments will directly provide the rationale for early phase clinical trials in patients. The above work will benefit academia in general, industrial collaborators, and ultimately, patients with cancer. In the short-term, we anticipate that the optimised cancer models will enhance our understanding of the interaction of tumour cells with immune cells in the tumour. We also anticipate short-term preliminary therapy results. These results can then be used in the mid-term to set up early phase clinical trials, which in the long-term are likely to lead to improved patient outcomes.

What outputs do you think you will see at the end of this project?

At the end of this project, we expect to have new information on: 1) the development of more informative tumour models; 2) the optimisation of both single and combination treatment strategies and their schedules of therapy; and 3) providing rationale for early-phase clinical trials. We also expect to produce peer-reviewed publications as a result of the undertaken work.

Who or what will benefit from these outputs, and how?

This work will be of benefit to academia in general, industrial collaborators and, ultimately, patients with cancer. In the short-term, we anticipate that the optimised cancer models used here will enhance current understanding of the interaction of tumour cells with the immune microenvironment.

We anticipate that the results from these experiments will provide the basis for early phase clinical trials, which in the long-term are likely to lead to improved patient outcomes.

How will you look to maximise the outputs of this work?

Results from this work will be publicised through peer-reviewed publications, as well as through oral and poster presentations at national and international conferences.

Species and numbers of animals expected to be used

- Mice: 3900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We plan to use mouse tumour models for our experiments, which constitute the lowest form of mammal recognised as being relevant to human cancers. To achieve the aims of the project, we will use mice at the adult stage of life to ensure we can study a fully-functioning immune system to provide meaningful data towards clinical trial applications in humans.

Typically, what will be done to an animal used in your project?

The vast majority of animals will be injected with tumour cells which will be allowed to grow and form established tumours. Animals will receive treatment with various drugs, including

immune-stimulating drugs, or radiotherapy. Tumours will be measured and examined in order to assess the effects of therapy.

What are the expected impacts and/or adverse effects for the animals during your project?

Injections of tumour cells and the resulting growth of cancer masses may cause some transient discomfort. Similarly, procedures involving treatment with various drugs or radiotherapy may cause transient discomfort to the mice, the majority of which will be from the administration of the treatment
e.g. needlestick.

Expected severity categories and the 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 animals are not expected to suffer more than a moderate degree of adverse effects during the entirety of our 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?

As far as possible, my group utilises patient-derived samples including blood and tumours for laboratory studies. These studies can yield some information regarding the effects of cancer therapies on tumour and immune cells. However, immune responses in patients involve complex interactions of multiple cell types in different sites within the body and these interactions cannot be fully replicated in a laboratory setting without the use of animals. A fully-functioning competent immune system is essential to evaluate new strategies for use in patients and hence there is an absolute requirement for animal studies.

Which non-animal alternatives did you consider for use in this project? Laboratory studies using patient-derived samples including blood and tumours. Why were they not suitable?

Immune responses in patients involve complex interactions of multiple cell types in different sites within the body and these interactions cannot be fully replicated in a laboratory setting without the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Statistical analysis will be used to determine the minimum numbers of mice used, while ensuring sufficient data are generated to produce meaningful results. The use of pilot studies will help to assess animal numbers and how best to design the main study in order to gain maximum information.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing experiments, statistical analysis, including power calculations, will be performed to ensure that we use the minimum number of animals per group to be informative and to provide robust results.

Our experiments will be conducted using genetically identical animals that are free of obvious disease; this avoids the variability of treatment response associated with non-identical animals and thus reduces the required sample size.

Initially, preliminary non-animal laboratory work will be used to guide therapeutic dosing and treatment schedules, which will be evaluated in pilot studies involving small groups of animals. These short-term studies will guide the most appropriate doses, schedules and combinations of therapies to be undertaken in longer-term experiments in both protocols 1 and 2. Group sizes for individual experiments will be based on efficacy seen in these preliminary experiments. For new models, study design will be discussed with statisticians prior to planning large experiments

We have utilised the NC3R's Experimental Design Assistant to plan experiments and sample size has been determined using Power calculations as determined using G*power 3.0.10.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To maximise the information from a single animal, we will aim to collect tissue samples from multiple body sites and provide other affected tissues to appropriate scientists, so that they do not have to breed mice specifically for their experiments.

Non-invasive imaging techniques in live animals and analysis of tissue samples collected after sacrifice of the animal will allow us to maximise data collection during and after experiments and reduce the total 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.

All of our experiments will be conducted in mice. Most of the experiments planned will utilise a superficial tumour model, which is a short-term model that does not require the added stress of anaesthesia for tumour measurements. The severity of the procedures used will be kept to a minimum, whilst providing meaningful data for progression of these approaches to patient care. None of the studies detailed in this project exceed a 'moderate' degree of animal suffering as useful data can be obtained from animals bearing low tumour burden, with the animals being killed after only short periods of tumour growth.

Why can't you use animals that are less sentient?

The use of less sentient animals or those terminally anaesthetised is not possible here due to the need for the whole animal physiologically to enable tumour growth.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All of our experiments will be conducted in mice. Mice constitute the lowest mammal recognised to be relevant for human cancers. Most of the experiments planned will utilise a superficial model, which is less intrusive than implantation of tumour into the liver and does not necessitate anaesthesia for tumour measurement, as is required for liver tumour models.

Animals will be monitored daily for any signs of distress that might affect their well-being. Such signs of distress include loss of appetite, huddling, shivering or any abnormal behavioural problem. Any mice that show persisting signs of distress will be humanely killed.

Superficial tumours may develop signs of ulceration, irritation or reddening. Animals developing such symptoms will be closely monitored and where needed pain controlled through use of analgesia.
Tumours will be monitored using a tumour scoring system and humane end-points will be based on a both tumour size as well as tumour ulceration in combination with general well-being of the animal.

In the majority of experiments, this subcutaneous model will be more than sufficient to determine the effectiveness of the treatments being used with minimal harm/discomfort to the animals. However, the most promising approaches (those that show the greater impact on tumour burden) will then be tested in the liver model to further confirm the relevance of these therapies before moving forward to clinical trials in patients.

The severity of the procedures used will be kept to a minimum, whilst providing meaningful data for translation of these approaches to patient care. Discomfort and distress experienced by the animals will be limited to unavoidable procedures required for the conduct of sound research. All methods will be based closely on UKCCCR Guidelines for the welfare and use of animals in cancer research British Journal of Cancer (2010) 102,

1555 –1577. For delivery of therapeutic agents, daily maximum volume and number of injections will not exceed the limits recommended in the guidelines above.

None of the studies detailed in this project exceed a ‘moderate’ degree of animal suffering as useful data can be obtained from animals bearing low tumour burden, animals being killed after only short periods of tumour growth.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All methods will be based closely on UKCCCR 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 will keep up to date with advances in the 3Rs by keeping up to date with correspondence regarding this and ensuring attendance of the PPL holder and any PIL holders working on this licence at the relevant meetings.

99. Elucidating the development and function of the chicken immune system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - 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

Chicken, Immunity, Transgenic, Gene editing, Immune response

Animal types	Life stages
Chicken	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to answer the question: As the basic structure of the birds' immune systems is quite different to mammals, how do chicken immune cells develop and perform the same basic functions of immune surveillance and antigen presentation as their mammalian counterparts?

To achieve this aim, this project will genome engineering technologies to study the development and function of the avian immune system

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Poultry, especially chickens, form the most important sector of livestock keeping worldwide, with the production of meat and eggs being a major contributor to human nutrition. Management of diseases is a major challenge to poultry production in terms of economic cost, animal welfare and, for avian influenza for example, the zoonotic challenges to human health. Increasingly, the improvement in protection against pathogens in poultry production must be achieved in the context of overall reduction of antibiotic use. Knowledge gains in avian immunology that lead to better and more efficient vaccination strategies or breeding of more healthy robust chickens will have significant academic, social, economic, environmental, and industrial impacts.

Unlike mammals, birds do not have specialised lymph nodes and have other very different lymphoid tissues. Despite this the majority of our knowledge on how vertebrate immune systems develop and function is based on mammalian model systems. To understand how the chicken system develops and functions and use this knowledge to improve animal health, we must first produce avian specific biological models. This project will enable the production and maintenance of gene edited and transgenic chickens which will be a very powerful way of generating robust biological models, and will lead to rapid knowledge gains in chicken immunity.

What outputs do you think you will see at the end of this project?

Output derived from this project will include: Knowledge gains in avian immunology

Publications on avian immunology

Novel gene edited chicken lines

Novel avian specific immune assays Improved vaccine design

Who or what will benefit from these outputs, and how?

Stakeholders have been identified as beneficiaries of this work:

The poultry breeding industry:

The consequences of improved vaccine responses and disease resistance may provide a panel of phenotypic biomarkers which could be developed as affordable tools to inform breeding strategy. We have established collaborations with major poultry breeding companies.

The animal health industry:

The data generated during this programme will allow us to improve vaccine targeting and will develop tools to modulate immune responses at mucosal surfaces.

Animal welfare:

The reduction of disease as a result of improved vaccine strategies supports the Five Freedoms implicit to animal welfare as set out by the Farm Animal Welfare Council.

General public and the environment:

The consequences of improved vaccine responses and disease resistance will lead to a reduction in the prophylactic use of antimicrobials and the risk of contamination of the food chain and the environment.

Academia and Training:

The multidisciplinary nature of this programme will provide opportunities for broad training to all staff including other members and students of the institution ('strengthen the research community in the areas of disease and pest resistance of farmed animals through interdisciplinary research and the provision of training'). Results with respect to the identification of cell subsets associated with antigen uptake, processing and presentation will be of interest to a wide scientific community and will be published in peer-reviewed journals and presented at national and international scientific meetings.

How will you look to maximise the outputs of this work?

Results will be shared primarily by publication. Owing to the high potential for major knowledge advancement; we will target general interest journals to reach the widest audience (e.g. Nature, Science, PNAS) and high-ranking discipline-specific journals (e.g. Journal of Immunology, Elife, Genome Biology). All papers will be open-access and made available . We will share our findings, including unsuccessful approaches, at international scientific conferences, engaging researchers working in diverse fields and informally through our laboratory website, our institutional websites and via social media.

Species and numbers of animals expected to be used

- Domestic fowl: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The use of chickens is appropriate as the development of new immunological reagents and vaccines requires an understanding of the immune system in birds, and extrapolation from rodent models is not appropriate as there are many significant differences between the immune system of birds compared to mammals.

The avian immune first forms in the early embryo and is present during the entire life of the chicken. Studying the chicken immune system will involve study of all chicken age stages, including embryos.

While a chickens lifespan can be as long as 7-8 years, broiler chickens are mostly culled by six weeks of age. As a consequence of this immunisation of chickens occurs in the late embryo stages (day of incubation 17-19) and in neonatal chicks. For these reasons, studies of the immune system of chicks of this age are required and appropriate.

Typically, what will be done to an animal used in your project?

Chickens used in this programme to study the development of and function of the avian immune system may have the following procedures done to them:

Genetic modification. Changes will be made to the individual base pairs, or genes. This may include deletion of genetic information or the addition of genetic information (e.g. transgenic chickens). These changes will be made in primordial germ cells or the very early embryo. These changes will be for the duration of the chicken's life.

Genetic modification may have no apparent impact on chicken development and health (e.g. a transgenic reporter chicken expressing a fluorescent reporter protein) or may impact the development and immune system of chickens (e.g. knockout of a immune related gene or immune cell type). These changes will be for the duration of the chicken's life.

Genetic modification to delete a specific gene, maybe done in such a way that it is acute and transient (one to two days) or could occur for the duration of the chicken's life.

Injection of chickens (genetically modified and wild-type control birds) with antigens. These antigens may include known model antigens or novel vaccine antigens. Antigens may be injected in a neutral medium (e.g. saline) or adjuvants to drive specific immune responses. The duration of these procedures would vary with particular experiments, as would the route of injection. Routes of antigen administration via injection may include: intravenous, subcutaneous, intra-muscular. Non-injection routes of antigen administration may include: Intratracheal, intracloacal, intranasal and intra-ocular.

Injection of chickens with substances that modify immune responses. Substances may include natural or recombination proteins (e.g. cytokines, chemokines or growth factors), pathogen derived substance (e.g. LPS or microbial derived nucleic acids), specific immune agonists (e.g. specific agonists to trigger cell death in genetically modified cells), or a combination of these factors. The duration of these procedures would vary with particular experiments, as would the route of injection. Routes of antigen administration via injection may include: intravenous, subcutaneous, intra-muscular. Non-injection routes of antigen administration may include: Intratracheal, intracloacal, intranasal and intra-ocular.

Cell transfer: Immune cells from non-genetically modified and genetically modified chickens (including embryonic and post-hatch stages) may be transferred into non-genetically modified and genetically modified chickens to determine how the immune system develops and functions. The duration of these experiments will vary with individual experiments, but may be for a very short period (e.g. one day) or for the duration of the animals life. The duration of these procedures would vary with particular experiments, as would the route of injection. Routes of antigen administration via injection may include: intravenous, subcutaneous, intra-muscular. Non-injection routes of antigen administration may include: Intratracheal, intracloacal, intranasal and intra-ocular.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse effects will depend on the nature of the procedure. In some cases (e.g. in genetically modified chickens) this will be for the duration of the chicken's life, while in other cases it will be of an acute nature (e.g. injection of LPS).

Impacts may include:

- increased susceptibility to infections.
- Loss of specific immune cell populations, rendering birds refractory to successful immunisation.
- developmental abnormalities. Genetic modification of chickens to study the development/function of the immune system, may impact other body system development and/or function.
- Weight loss. Increased susceptibility to infection or developmental abnormalities can result in weight loss.
- Pain. Increased susceptibility to infection or developmental abnormalities can result in pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity of the procedures is expected to be moderate.

The severity limit for most gene edited chicken lines is expected to be mild.

In cases where genetic modification procedures effect immune function, the severity limit may reach moderate levels.

Injection of antigens or immune modifying substances will be a moderate severity level.

On balance, the proportion of minimum to moderate severity for these procedures is expected to be at a ratio of 70:30.

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?

Poultry form the most important sector of livestock keeping worldwide, with the production of meat and eggs being a major contributor to human nutrition. Management of diseases is a major challenge to poultry production in terms of economic cost, animal welfare and, for avian influenza for example, the zoonotic challenges to human health. Increasingly, the improvement in protection against pathogens in poultry production must be achieved in the context of overall reduction of antibiotic use. Knowledge gains in avian immunology that

lead to better and more efficient vaccination strategies or breeding of more healthy robust chickens will have significant academic, social, economic, environmental, and industrial impacts.

The use of chickens is required to study the development and function of the avian immune system as there are fundamental differences between the mammalian and avian immune systems. For instance, birds do not have lymph nodes which are a highly developed feature of the mammalian immune system. This in turn means that knowledge derived from mammalian model systems cannot simply be used it infer information about the avian immune system.

Gene editing of chickens is a methodology that will enable the creation of high quality biological models that will enable the rapid knowledge gains in the developmental biology of the chicken immune system and an overall reduction in the number of chickens used, compared to conventional techniques.

Which non-animal alternatives did you consider for use in this project?

We considered trying to grow different chicken immune cells so they could then be studied in cell culture. Potentially we could change the genes in these cells to study gene function.

Why were they not suitable?

The number of chicken immune cell types that can be grown in culture is extremely limited and the ability to culture and gene edit keep immune cell types is lacking. Immortalised chicken cell lines for some key immune cell types (e.g. T-cells and dendritic cells) also do not exist. It is also not possible to address the key aim of this project ("How do chicken immune cells develop and perform the same basic functions of immune surveillance and antigen presentation as their mammalian counterparts in a radically different lymphoid tissue environment?") using cell culture as this cannot replicate the complexity and diversity of chicken lymphoid systems.

Our skill in culturing chicken macrophage cells allows us to carry out some experiments in cell culture without using animals. Our use of chicken embryos mostly involves injecting macrophages at unregulated young stages in eggs and many of the experiments will also be performed before hatching of the chick. The use of the chicken as a model species is an improvement over using mammals. In this instance, the chicken offers advantages over using a mouse models as the mother does not need to be killed to obtain the embryo and a surgical manipulation is not needed to introduce donor reproductive cells into an embryo. The majority of experiments will also be carried out at unregulated developmental stages (before day 14 of incubation).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 chicken, we have estimated the total number of genome edited and transgenic lines we will establish over 5 years, the number of genome edited offspring that will be bred during that time period. For each genome edited line, we will need to generate surrogate host birds that will carry the genetic material. For each experimental genome edited line, we use a power calculation to determine the number of animals we need to breed in order to carry out the required experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The cell culture of some immune cells types (e.g. macrophages) enables us to carry out some experiments without using animals. For the production of novel gene edited chicken lines the injection of lentivirus pseudovirus or donor reproductive cell are carried out at unregulated developmental stages in eggs and many of the experiments will also be performed before the chick would hatch. The production of novel transgenic immune cell reporter lines of chickens reduces the number of animals used overall as if offers a highly efficient model to visualise and characterise specific immune cell populations compared to conventional techniques.

The use of avian species as models offers advantages over mammalian species for the study of the early stages of the development of the immune system. In this instance, the chicken offers advantages over using a mouse models as the mother does not need to be culled to obtain the embryo and a surgical manipulation is not needed to introduce donor reproductive cells into the embryo. The majority of experiments will also be carried out at unregulated developmental stages (before day 14 of incubation) in the egg.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

With our colleagues at our Institute we have worked to improve the efficiency of hatching surrogate chicks carrying the donor genome edited reproductive cells. Colleagues have developed germ cell culture and genetic screen techniques that in combination with genome edited surrogate hosts that mean that 100% of the offspring are derived from the donor genome edited cells. This means that compared to older methods, there are no excess non-GA bird produced. We are also developing methods to reduce breeding by directly mating the surrogate hosts to generate pure offspring in a single generation which greatly reduces the number of chicken bred in these 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 use the chicken as a model to study the development and function of the avian immune system. The use of lentivirus transgenesis of early embryos or cultured avian

reproductive cells that can be genetically modified to disrupt gene function and used to produce genome modified birds is unique to the chicken and avian species.

The production of transgenic immune cell reporter chickens (e.g. the expression of a fluorescent reporter protein in selected immune cell populations) is not expected to cause any pain, suffering, distress, or lasting harm to the animals.

In the case of gene edited birds in which a particular gene is knockout out to access the impact of the gene product on the development and/or function of the immune system, we are refining systems that mean that this will result in temporally or cell specific restriction of gene knockout to minimise pain, suffering, distress, or lasting harm to the animals.

The studies are designed to be as minimally invasive as possible; the vast majority of birds will only be blood sampled to confirm transgenesis and/or gene editing and then used for breeding as required. All new genetically modified lines are closely monitored for any adverse phenotypes. We work closely with our named veterinary surgeons.

Why can’t you use animals that are less sentient?

The chicken is an important agricultural animal and it is important to study the development and function of the immune system of this animal for sustaining and improving food production. The understanding of the genes involved in the major production traits of disease resistance, feeding, and production traits will lead to more efficient agriculture and the production of more healthy, robust poultry.
The biology of immunology of birds is of interest in terms of understanding evolution of the immune system and function of individual immune cell types of all vertebrate animals (including humans).

Applications of gene editing technologies in genetics and immunity also must relate to the function of the intact animal. As the chicken is used as a livestock species for meat and egg production, studies using these bird will largely be focuses regulated embryonic stages (post day 14 of incubation) or post- hatch stages. For studying the early development of the immune system in vitro culture techniques and/or embryos at unregulated developmental stages (before day 14 of incubation) in the egg will be used as appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are developing gene editing technologies which will enable the temporally and immune cell lineage specific manipulation of gene function. The aim of this refinement will be to narrow the impact of gene manipulation to produce more precise, high quality data on gene function and to reduce pain, suffering, distress, or lasting harm to the animals.

We have instigated monitoring the weight of our newly hatched genome edited chicken to verify a normal growth curve for hatchlings.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All work is undertaken in close collaboration with our Named Veterinary Surgeons. Study protocol forms must be completed and submitted to them prior to proceeding with any

experimental work and animal unit staff are closely involved in experimental work and will offer advice on improvements to experimental protocols and monitoring/raising of the animals.

The group contains all the competencies required to perform the majority of techniques and procedures outlined in this project. Where additional expertise is required, staff will undergo appropriate training until competent.

Animals will be kept in accordance with annex III of Directive 2010/63 EU and/ or Home Office Codes of Practice, as appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We communicate with our NVS on current developments to enrich the environment of housed chicken. Our poultry workers are trained in proper welfare techniques for birds and we are strictly monitored by our veterinary scientists and the Home Office on current practices. We visit the NC3R website for new information on animal use in research. We hold annual conferences in veterinary ethics and welfare.

100. Dissecting the response to metabolites in the inflammatory micro-environment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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-mediated inflammatory diseases (IMIDs), inflammatory disease micro-environment (IDME), ectopic lymphoid structure (ELS), metabolic pathways, immune response

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 this project is to identify the metabolic type of immune cells in inflammatory diseases that are characterised by the presence of ectopic lymphoid structures (ELS), collections of immune cells in specific parts of the body that contribute to the development of diseases including Sjogrens syndrome and Rheumatoid arthritis. This information will potentially identify new ways to treat these diseases by changing the cell's metabolic type to one that is anti-inflammatory.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 open new possibilities for drug development by aiming to change the

metabolic type of immune cells to one that is anti-inflammatory. In the longer term, this will impact positively upon the clinical management of chronic inflammatory diseases such as Sjogrens syndrome, Lupus, Multiple Sclerosis and Rheumatoid arthritis, which all involve the development of ELS.

What outputs do you think you will see at the end of this project?

We will generate new information on the role that the metabolism of immune cells, and metabolic products such as lactate, play in the formation of ectopic lymphoid structures (ELSs) in inflammatory conditions such as Sjogren's syndrome, Lupus, Multiple Sclerosis and Rheumatoid arthritis. These ELS are known to help maintain the inflammation in these diseases. The successful delivery of this research programme is likely to result in high impact publications benefiting the scientific community at large.
The new information in this project on metabolism and immunity may also encourage new thinking in other diseases that are driven by uncontrolled inflammation, such as cardiovascular disease.

Who or what will benefit from these outputs, and how?

We have chosen Sjogren's syndrome as a paradigm for inflammatory conditions that involve the formation of ectopic lymphoid structures as a key part in disease pathogenesis. The murine model we have chosen, the sialoadenitis mouse model, recapitulates very well the clinical features of this disease in humans and will allow us to carry out research that will give short, medium and long term benefits as follows:

Short term gains: From the start of the project a range of communities will benefit as we will share any knowledge generated with the wider scientific, clinical and pharmaceutical industry communities through attendance at conferences where we will present the data, and through publication of the data in appropriate journals using the ARRIVE guidelines on reporting in vivo experiments. We will also ensure that refinements that we develop over the course of the in vivo work are also reported as part of our commitment to the 3Rs. Additionally, we will be engaging with established Public and Patient Involvement and Engagement (PPIE) groups to ensure our communications are suitable for a lay audience.

Medium term gains: We anticipate the main outputs from this work to be the scientific data and associated publications, and the identification of novel drug targets for chronic inflammatory diseases such as Sjorgens syndrome. We disseminate our work frequently at conferences and have been invited to speak about our work in more than 20 occasions in the last three years.

We would also envisage the submission of a patent that would cover the “use of any novel agents in the treatment of chronic inflammatory conditions” and would benefit the pharmaceutical industry by identifying a potential new therapy.

Long term gains: Our work in this area will advance scientific knowledge of the processes regulating the immune response during chronic inflammation and specifically in diseases that involve generation of ELS. In particular, it will highlight to what extent these processes depend on metabolism and metabolites such as lactate. In this way, it will help to identify new strategies to modulate these processes, which are relevant not only to Sjogren's syndrome but to many chronic inflammatory conditions for which altered metabolism is a driver, including Rheumatoid arthritis, Multiple Sclerosis, Myasthenia Gravis and Lupus.

Overall, given that the programme of work is built upon previous preliminary data and the published literature, we have confidence in our approach and the likelihood of its success.

We have expertise in the model proposed, the sialoadenitis mouse model, both in our team and through our external collaborators. This gives us further confidence that skills exist and will be developed further to run the sialoadenitis model. We do not anticipate any issue we will not be able to deal with in compliance with the health of the animals.

How will you look to maximise the outputs of this work?

The proposed research will benefit other researchers in the fields of immune metabolism, autoimmune and musculoskeletal diseases, as well as those in other disciplines of medicine for the following non-exhaustive reasons:

Acquisition of knowledge.

We will provide novel information to researchers in the emerging topic of how metabolism influences immune cell function, particularly switching them to a pro-inflammatory type, contributing to immune- driven disease.

Specifically, we will aim to provide a significant advance in understanding of the role of one metabolic product, lactate, in rewiring of immune cells towards a pro-inflammatory type, ectopic lymphoid structure formation and autoimmunity. We will focus on Sjogren's syndrome, a common autoimmune disease whereby ectopic lymphoid structures form in the salivary glands and impact on disease severity. These ELS also increase the risk of developing a type of cancer termed lymphoma.

Overall, this programme of work will not only benefit researchers in the specific field of Sjogren's syndrome but has the potential to significantly impact on other autoimmune diseases such as Rheumatoid arthritis and Lupus. In addition, the formation of ectopic lymphoid structures can also occur in certain infections such as TB and cancers. Consequently, our research has the potential to benefit several areas of medicine.

Benefits of promoting interactions among scientists across different fields of research.

The identification of the processes which link metabolic changes occurring at sites of inflammation with the development of autoimmune conditions such as Sjogren’s syndrome, will be achieved through interactions between scientists from different backgrounds including biochemistry, molecular biology and immunology. In addition, expertise in translating research from robust animal models to confirmation of clinical relevance via human biological samples is of critical importance.

Benefits of strengthening successful collaboration in the UK.

The successful delivery of this research programme is likely to result in high impact publications benefiting the scientific community at large. In addition, the successful delivery of this programme of research would generate novel thinking in relation to other diseases and strongly encourage new collaborations between researchers.

Species and numbers of animals expected to be used

- Mice: 2,500 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 generation of ectopic lymphoid structures, as occurs in Sjogren's syndrome, is central to several autoimmune inflammatory diseases. It is a complex process which involves many cell types and occurs in several tissues; hence it can only be understood by analysing the process in a whole organism.

The mouse is the choice of species for our experimental models as they are well characterised physiologically and the induction of sialoadenitis in mice recapitulates the key features of human Sjogren's syndrome. This model has been used extensively to understand disease processes and has also been used to develop new therapeutic options. Lower animals in the evolutionary scale have insufficient complexity and their immune system is not sufficiently evolved to mirror the human context.

We will use animals between young adulthood to adulthood as animals of this age will more closely represent the onset of such diseases in humans.

Typically, what will be done to an animal used in your project?

On a maximum of four occasions, under general anaesthetic, animals will be administered with a replication deficient adenovirus or control into one or both salivary glands via a cannula.

Administration of antibodies or vehicle control by intravenous or intraperitoneal injection for up to once every three days for a maximum of 3 weeks. All volumes will be in accordance with LASA guidelines.

At any stage animals may be humanely killed OR under terminal anaesthesia have blood withdrawn and subsequently killed using a humane method.

What are the expected impacts and/or adverse effects for the animals during your project?

Cannulation: a slight swelling of the salivary glands is expected post cannulation. All animals will be given analgesia to minimise discomfort. Weight will be monitored (twice/day for the first 72 hours) to ensure the animal is able to eat and drink normally. In addition, a soft diet will be provided to aid food consumption. Any swelling should resolve within 48-72 hours post cannulation.

Any animal that fails to recover fully from the anaesthesia will be humanely killed.

Any animal where there is persistent swelling or is unable to eat and drink normally 72 hours post cannulation will be humanely killed.

Administration of antibodies: piloerection, shivering, reduced motility, hunched posture, subdued behaviour and reduced peer to peer interaction and mild weight loss might be observed. This should not last for more than 6 hours and not occur in more than 5% of animals.

These adverse effects are expected to be transient with mice returning to normal behaviour within 6 hours of agent application. Mice showing adverse effects will be treated with interventions such as a warming chamber and softened food and will be monitored for the 30 minute post injection of these compounds, then at least twice within the 6 hours post injection.

If any of the immediate adverse effects do not resolve (>6hr), 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)?

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?

Our aim is to understand how metabolism influences the development of ectopic lymphoid structures, collections of cells that are important in autoimmune diseases such as Sjogrens syndrome. Although experiments that do not involve animals, such as work on immune cells isolated from patients, have been informative to date, they can only take us so far. The processes involved in the development of ectopic lymphoid structures are highly complex and remain poorly understood. This is because multiple physiological processes and different cell types are involved, and importantly the environment that these processes occur in influences how the immune cells behave. Therefore, to understand these disease processes it is necessary to study them in a whole organism that replicates the human disease.

Which non-animal alternatives did you consider for use in this project?

We routinely perform assays with immune cells (e.g. T cells) and tissues isolated from humans or non- procedural (humanely killed, i.e. a partial replacement) mice for functional or molecular studies.

Where possible, cells or tissues from patients or healthy volunteers are also used to address questions relating to the mechanisms of action of therapeutic targets.

In addition, whenever possible cells or tissues from non-procedural mice (humanely killed) will be used for specific assays. For example, to obtain lymphocytes for in vitro assays we

will use cells which are isolated from these mice.

Molecules shown to be inactive in these assays will not be examined further in vivo to minimise our use of animals.

This utilisation of in vitro assays is an important replacement of in vivo experiments, but for other aspects of the research, such as how a cell behaves in the complex environment of the body and during development of ectopic lymphoid structures, we must progress to in vivo animal work.

Why were they not suitable?

Even though these in vitro experiments are important and will be informative throughout the project, ultimately the steps leading to the development of ectopic lymphoid structures (ELS) can only be addressed in a whole organism. For example, understanding how the lactate produced in the salivary gland influences immune cell inflammatory state and the recruitment of other cells to form the ELS cannot be done ex vivo as it cannot recapitulate the situation in the body.

We will continue to review the scientific literature on a regular basis in order to identify any newly emerging technology and models that could be potentially adopted to address our experimental aims and replace in vivo animal use. To this end, if undertaking a systematic review, we will use SyRF. Furthermore, by regularly checking the NC3Rs website we will keep up to date with any improvements in protocols and techniques which may reduce or replace the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

Numbers of animals required are based on good colony management, previous experience of running these experiments, pilot data and regular consultation with statisticians in the Institute. All models and protocols proposed in this application have been extensively validated and optimised by the wider inflammation research community. This is important because it will reduce the number of animals required, and the need to set up and optimise experimental protocols. An important goal in our programme of research will be to identify any sources of inter-laboratory variation to improve reproducibility of key observations in the field of inflammation biology.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In vitro methods will be used initially in all cases to test the efficacy of compounds, prevent the use of ineffective treatments in animals and estimate the magnitude of the expected response.

The literature will be continually reviewed to ensure that we are not unnecessarily

repeating published work and that our hypotheses and procedures are based on the most up to date knowledge.

Where pilot data exist, we will always perform statistical calculations to ensure that we use the minimum number of animals needed to obtain significant results. In designing our experiments, we aim to ensure randomisation of animals to different treatment groups across the different cages, and we will try to ensure that people performing analyses are blinded to animal treatment groups to prevent confirmation bias.

We will use the G*Power statistical analysis package (www.gpower.hhu.de/en.html) and the NC3R's experimental design tool to aid experimental design (https://www.nc3rs.org.uk/experimental-design- assistant-eda) and consult trained statisticians before using any new protocols.

All staff performing animal experiments will attend appropriate training on key aspects of experimental design and will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment. We will also look to publish in Open Access Journals, wherever possible and in accordance 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?

In PPLs, such as this one, that involve the use of transgenic animals to address specific research questions in vivo, it is important to optimise breeding strategies to obtain enough transgenic animals and appropriate control animals in a timely fashion. We will thus follow recent guidance notes from the Home Office Inspectorate and take advice from our colleagues, our NACWOs and our NVS.
Whenever possible, the same mouse will be used as experimental and as control individual. However, since most of the effects we will induce and observe are systemic, we will need to make use of separate control and experimental groups in most cases.
Whenever possible, untreated control groups will be shared between treatment groups. In addition, untreated control groups will be used as a source of tissues for analysis.

Where new routes of administration or new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments to minimise testing of ineffective treatments. In conjunction, we have optimised methods of tissue and data collection from individual animals ensuring that the numbers used are sufficient to address all of our research goals whilst providing biologically significant results that would not require further experimental repeats.

All experiments will be carried out according to 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.

It is vital to utilise experimental models that most closely mirror the human disease so that effective therapies in these models are likely to translate into novel treatments in the clinical setting. The mouse is the choice of species for our experimental models as they are well characterised physiologically and immunologically. In mice, the induction of different inflammatory diseases that mimic human disease has been demonstrated extensively and these models have been used to successfully develop new therapeutic options.

The model of sialoadenitis (recapitulating key pathological features of Sjogren's syndrome, including inflammation of the salivary glands) has been developed, validated and optimised by our collaborators. This is important because it will reduce the number of animals required, and the need to set up and optimise experimental protocols. This is our model of choice over other possible models that develop ectopic lymphoid structures (ELS), such as murine models of arthritis, for 2 main reasons: 1) 100% occurrence of ELS (instead of 60-70% of the most widely used model of arthritis, i.e. collagen-induced arthritis; 2) ELS, our key study target (central to Sjogren's syndrome pathology), are particularly well defined in this model and allow us to more effectively assess the effects of interfering with their formation and function.

Overall, the protocols will carry a moderate risk to the health of the animals in the experiments. We have made refinements to the housing of the animals to cater for any disability arising from the pain/swelling of salivary glands due to the cannulation, including food on the cage floor and long drinking spouts on water bottles.

In all protocols we will aim to utilise approaches that minimise the animals' potential suffering. The likelihood of adverse responses will be minimised for agents by choice of dose rationale informed by prior in-house pilot investigations and in vitro studies and experience from collaborators or published data.

During all our pilot work we monitor the adverse effects very thoroughly which enables us to refine the main experiment accordingly. Similarly, after every experiment we critically review it to see if there are any welfare gains that we could apply to the next experiment.

Why can’t you use animals that are less sentient?

We have selected the mouse for our research species as they are very well characterised in relation to their immune system and physiology. Importantly, in mice, the induction of chronic inflammatory diseases that mimic human disease is well established and these models have been useful in developing new treatments. The anatomy, physiology and immune responses of lower species do not sufficiently mimic those of mammalian species and thus a complex disease such as Sjogren’s syndrome cannot be modelled in these species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local AWERB. Following every experiment and regularly

during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

In all protocols we will aim to utilise approaches that minimise the animal's potential suffering. The likelihood of adverse responses will be minimised for agents by choice of dose rationale informed by prior pilot studies and the experience of our collaborators, and the scientific community as published in the literature.

During all our pilot work we monitor the adverse effects thoroughly which enables us to refine the main experiment accordingly.

Overall, the protocols will carry a mild to moderate risk to the health of the animals in the experiments. We have made refinements to the housing of the animals to cater for any disability arising from the protocol including food on the cage floor, long drinking spouts on water bottles.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All staff involved in animal experiments will make use of LASA dosing/blood sampling guidelines, will review the literature on animal welfare provided by the local AWERB, as well as routinely refer and check ARRIVE guidelines and NC3Rswebsite.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will check regularly NC3Rs website, sign up for their newsletter and attend relevant courses offered by NC3Rs for best practice with animal work. We will publish all in vivo data in open access journals and in accordance with the ARRIVE guidelines for reporting.

101. Investigating the pathobiology and therapeutic opportunities of ubiquitin ligases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, Parkinson's disease, mitochondria, protein homeostasis, therapeutics

Animal types	Life stages
Mice	adult, pregnant, 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?

The aim of this project is to discover the roles played by proteins, known as ubiquitin ligases, in different cell types and how their malfunctioning causes diseases and test whether they can be used to cure or treat disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ubiquitin ligase are cellular proteins of fundamental importance in the cells of our body. They are enzymes that modify other proteins and in doing so, they regulate many different processes that control the health and proper functioning of cells. The poor functioning of these enzymes is at the root of many different diseases, ranging from cancer to neurological diseases to infertility. By understanding the functions of ubiquitin ligases, we

can discover a way to prevent or treat these very different diseases.
Ubiquitin ligases are remarkably specific and influential in their actions within the cell. We will investigate whether they can be used in disease settings to help reduce or cure disease.

What outputs do you think you will see at the end of this project?

The outputs we expect to create include new knowledge in understanding how a class of proteins, known as ubiquitin ligases, affect the ability of cells to control their metabolism and the production of their proteins. We expect also to see how defects happening in a small population of cells affect the overall health of the animal. We will also discover how new chemical and biological treatments designed based on the biology of ubiquitin ligase can change how a disease happens in the body. We anticipate this will lead to publications in scientific journals as well as potentially to new products that could lead to new treatments for patients. We could provide data to support the design of future clinical trials for patients with parkinsonism or with Parkinson's disease.

Who or what will benefit from these outputs, and how?

The main beneficiaries of these outputs will be the scientific research community who work on disease mechanisms of different diseases, including cancer and Parkinson's disease. In the short term, publications will inform the direction of other research projects and drug discovery approaches centred around ubiquitin ligase biology. Our work will also aid the clinicians who treat patients with a family history of Parkinson's disease. In the long term, we aim to use our knowledge to design therapies arising from ubiquitin ligase biology.

How will you look to maximise the outputs of this work?

We make our results available in a timely fashion in Open Access publications, and our research datasets are deposited in publicly available and searchable databases, linked to our laboratories website and our institutes' data repositories. We will also publish our preprints on biorxiv, which makes our work publicly available even before peer review. Our results will include negative and unexpected experimental findings. We have also presented our work at national and international conferences and use these opportunities to collaborate and share results and ideas with other researchers in similar or overlapping research fields.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice to study what happens in specific tissue types when they lose expression of a ubiquitin ligase. It is not possible to study this effect in a simpler system because the cellular processes we are studying are complex. These include the production of sperm and the effects of nerve cells dying on a mouse's movement and

coordination. There are no cell culture systems that replicate the sperm-producing capacity of the testes. Starting in adolescence, people with mutations in the gene encoding a ubiquitin ligase develop a progressive parkinsonism which affects their coordination along with other symptoms. The nerve cells of mice that do not have this gene will eventually die, and the mice will experience movement deficits slowly between 5-30 weeks of age. Therefore, these mice can be used to study some features of the human condition. Moreover, these mice are unique, as these clinical signs are not observed in less sentient animals.

Typically, what will be done to an animal used in your project?

Mice will be specially bred to bring together particular combinations of genetic mutations in ubiquitin ligases which may lead them to develop diseases. Mice may be injected in a surface vein with viruses containing a specific gene to test for the ability of that gene to slow or stop the death of cells. Neither the injections nor the viruses cause diseases but are instead aimed at treating the diseases that result from the mutations in ubiquitin ligases. These diseases typically take between 5-30 weeks to manifest, and some mice will be injected during this time frame. Animals will usually undergo one procedure, usually being genetically modified, and approximately 75 mice will also be injected with viruses, undergoing two procedures.

Mice may have tumour cells injected into them under their skin to allow a tumour to grow. These tumours may be treated with new therapies delivered by injection, either in a surface vein or into the tumour, to slow or stop the tumours from growing. These experiments normally take between 4-12 weeks, with tumours taking about 2-8 weeks to develop, and injections taking place over 1-2 weeks after tumours are established. A maximum of 100 mice will undergo one procedure of being injected with tumour cells and allowing tumours to grow. Of these animals, about 50 may be injected with a therapeutic based around ubiquitin ligase biology to test the ability of slowing or stopping tumour growth. Others will be controls.

Mice will be humanely killed using several methods. Different methods are used so we obtain brain tissue ideally suited to use in laboratory tests (*in vitro*) once the animal is dead.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the animals on this licence will experience no adverse or mild effects. Some mice with loss of a ubiquitin ligase limited to specific cell types will develop mild defects with coordination. A few mice will develop tumours, as we test novel agents for their ability to stop or reduce tumour growth.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice Mild 38%
Moderate 62%

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?

It is possible to generate some types of information about how some aspects of organ (e.g., brain) and tissues develop using cell cultures. However, it is not possible to fully mimic the normal and disease process in the laboratory (*in vitro)* as these experimental systems do not fully replicate the complexity of cell interactions or disease processes seen in an organ or a whole animal (*in vivo)*. At present, we cannot use cultured cell systems to ask how a particular protein functions in complex organs such as the brain, where multiple different types of cells exist and interact to enable the organ to function. Mice have similar developmental processes to humans, so to understand the normal and disease processes in complex tissues, it is appropriate to use mice where they can effectively model aspects of human diseases. Similarly, it is not possible to study tumour biology, and the complex contributions of the tissues surrounding a tumour to its survival in cell culture systems. For this reason, a tumour model is necessary. Also, to develop therapeutic approaches with the potential to treat human diseases it is necessary to establish their efficacy and toxicity in our animals. We will remain alert to any advances that will enable the replacement of animals in our work.

Which non-animal alternatives did you consider for use in this project?

We will, where possible, gather as much data as possible using *in vitro* assay systems. This may include more tractable cell culture systems, for example, that model organs by allowing growth in three dimensions or stem cells differentiated to neurons. We will also use patient skin cells to study aspects of cell biology and, where possible, reprogramme these to nerve cells, to address their specific functions, like growing specialised nerve structures, called axons. In parallel, we will utilise gene- editing technologies to introduce mutations into human and mouse stem cells in culture to make greater numbers of nerve cells, which reduce the need to generate new lines of genetically altered mice.

Why were they not suitable?

The induced neuronal cell models are not very efficient, and do not always differentiate to the specific sub-type of neuron that dies in Parkinson's disease and which we aim to study. Also, none of the *in vitro* models reflect whole animal physiology or neurodegenerative processes, e.g., like the impaired movement or the loss of connections between different regions of the brain which we see in the mice. There are no cell models that recreate the processes required to create the final shape and functionality of sperm cells. It is not possible to study these dynamic processes in cultured cell lines.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 mice to investigate aspects of Parkinson's disease require the use of mice bred with genetic changes. A number of genes must be inherited simultaneously in a mouse for the expression of a gene to be lost in neurons. Based on this, approximately 1 in 8 pups born will lack expression of the ubiquitin ligase in neurons. We estimate our numbers based on 5-6 breeding pairs having 5-6 litters of approximately 5-6 pups to generate mice for experiments. For mice experiencing infertility due to loss of gene expression, 1 in 4 pups born to a breeding pair will lack gene expression and only half will be male, so 3 breeding pairs is sufficient to generate mice for these experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have studied these mice for 5 years and found the clinical signs to appear in 100% of the mice that have mutated ubiquitin ligase genes, which reduces the number of mice needed for studies to generate statistically significant data.

We will perform pilot studies testing any new therapies in very small groups of normal mice. These new therapies will have already been tested in isolated cells in the laboratory. These small studies will allow us to determine the effectiveness of new therapies on tumours and minimise the suffering of these animals. We consult the advice offered in the PREPARE guidelines (https://norecopa.no/PREPARE) for planning our experiments.

We also use resources for study design on the NC3Rs website, which includes the experimental design assistant, and advice on conducting pilot studies, reporting, and 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?

Efficient breeding is the main measure that we use to reduce the number of animals used. Breeding pairs will be replaced after 6 litters and those with small litter sizes or husbandry issues will be replaced. Where possible, we will share tissue and mice with other labs that want any material from us, as we have done in the past.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 plan to test the functions of ubiquitin ligases with clinical relevance (i.e., where analysis will directly impact our understanding of human diseases). We will use two different genetically altered strains of mice for these studies, one that results in reduced expression in all cells of the mouse and the second limits this loss only to specific neurons. In both strains, most mice do not experience any adverse effects and a small number of mice will develop mild or moderate clinical signs as they age.

We also plan to test the effectiveness of new therapies based around ubiquitin ligase biology in tumour transplantation models. The mice used are well-established and allow for the measurement of multiple characteristics of tumour biology, including the capacity of the cancer to spread throughout the body and the growth of new blood vessels within the tumour.

Why can't you use animals that are less sentient?

We need to use adult mice since the neurological signs develop over time, starting at 5 weeks and continuing to 30 weeks of age. Additionally, male mice only become sexually mature at 10 weeks of age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All mice used under this license will be monitored for signs of pain and distress and inspected frequently for clinical signs, which will be recorded on score sheets. This will take account of things like appearance, posture, and behaviour. Tumour size will also be inspected, palpated, and measured by calipers and recorded. Mice weights will also be monitored and recorded.

If any clinical signs are found, mice will be promptly treated wherever possible (for example, with the use of different diets, analgesics for pain relief, and/or antibiotics), and if suffering cannot be promptly alleviated, mice will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Much of the guidance we use is based on our experience of our mouse lines, but also on the best practice resources for genetically altered animals on the NC3Rs website (https://www.nc3rs.org.uk/breeding-and-colony-management). We also seek advice from our NVS, NACWO, and the animal technicians in our facility who have regular contact with our animals. The animal experiments in our papers are described in accordance with the ARRIVE guidelines. The guidance documents and Position Papers from LASA on Transgenics, Education and Training, the 3Rs and the Concordat on Openness and Workman et al., 2010, *Guidance for the welfare and use of animals in cancer research*, are all relevant to my work.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am registered for monthly updates from the NC3Rs website, which is a major resource for

advances in the 3Rs and can consult the Laboratory Animal Science Association (LASA) guidance documents. I will also be in regular contact with members of my Project Support Team, which includes my NVS (Named Veterinary Surgeon), NACWO (Named Animal Care & Welfare Officer) and NIO (Named Information Officer). I can also consult resource pages on the NC3Rs website (https://nc3rs.org.uk/resource-hubs) and 3Rs in-house search tools and Norway's National Consensus Platform for the advancement of the 3Rs (Norecopa: https://norecopa.no/databases-guidelines), an external resource that maintains 10,000 pages of information and searchable databases about 3Rs strategies.

Home Office

102. Avian malaria across a migrant songbird distribution

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

avian malaria, distribution, migration, conservation

Animal types	Life stages
Pied flycatcher (Ficedula hypoleuca)	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 at understanding host-pathogen interactions in avian malaria, with a special focus on the role of insect vectors, and the combinations of host, vector and parasite genotypes in promoting pathogen transmission in different European populations of Ficedula flycatchers. These questions are studied using long term monitoring and data collection, as well as blood and insect vectors sampling of pied and collared flycatchers across their natural breeding range.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Migrants are amongst the most threatened of all bird groups; climate change and habitat change are all thought to play a role but our understanding of their importance and how they might interact is limited. Changes in climate and habitat are happening across the extensive ranges of migratory species, altering interactions between birds and their parasites, one of the most common being blood parasites, which could have beneficial or negative impacts. Migratory birds can have complex patterns of infection, for example

different blood parasites in different parts of the breeding range and different parasites in wintering areas. This is further complicated by breeding populations mixing in wintering ranges. To be able to understand the demographic and fitness consequences to populations of avian malaria caused by many blood parasites, and how these may change under different climatic and habitat change scenarios, we need to understand where birds become infected and monitor their effect. This project sets out to understand the distribution of avian malaria in a species that breeds across Europe and annually migrates to western sub-Saharan Africa.

What outputs do you think you will see at the end of this project?

The main outputs will be new knowledge on the relationship between host and vector genotypes and parasite load, which will be the subject of peer-reviewed scientific papers. The results may also contribute to guiding and prioritizing future conservation research, if patterns indicate population level consequences.

Who or what will benefit from these outputs, and how?

The major beneficiaries in the short and long term are likely to be other scientists with an interest in the global distribution of avian blood parasites, the study system and migration. Another potential beneficiary after the project is concluded is conservation of migratory songbirds, through knowledge gained on infection patterns and locations guiding where decline diagnosis and any interventions that address causes is best undertaken.

How will you look to maximise the outputs of this work?

I and my collaborators are regularly invited to present this work at national and international departmental seminars, conferences and workshops and we will use such events to disseminate the results of our work. This pathway will provide benefits to the academic community in the short term and allow us to publicise the work in its early stages.

Findings of wide interest will be publicised through press releases and social media from the University Press Office. We will author media-friendly articles for blogs and popular science magazines, and to citizen science audiences through specialist publications and blogs. Through our past research we have been very successful in promoting science to the wider public and our work has received considerable media coverage in the quality press, on radio and television, and we will with support from the University and RSPB media teams, ensure that results will be disseminated to the widest possible audience.

Due to the extensive collaborative network involved in this project, our work benefits from all collaborators across Europe similarly and simultaneously promoting and disseminating this work, thereby reaching a large and diverse audience. Past projects have led to new collaborations, both within the same network and with new research groups. Due to our data being included in the open source SPI-Birds database, the data is visible via a searchable web-based tool for other researchers to use on request.

Because we study individuals' breeding attempts it will also possible to measure the reproductive benefits or costs associated with different parasite prevalence, and we hope to be able undertake this additional analysis either ourselves or through collaboration.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The research will investigate the prevalence of avian malaria across the European breeding distribution of the Pied flycatcher. The species we have chosen is a good model to study avian malaria as they breed at high densities in nestboxes where they can be captured easily and enable breeding parameters and breeding-site vectors to be closely studied. The UK represents the north western extremity of the species breeding range and so provides geographical and climatic contrasts to other breeding populations across Europe included in the study. Our UK study population is representative of other UK populations and benefits from 65 years of collection of breeding and individual bird life-history data.

We use adult individuals as they can become infected with malaria either in the breeding areas (UK), on migration at stop-over sites or in the non-breeding (wintering) locations in Africa. In contrast nestlings can only be infected in the nest where they spend only 16 days, and due to the incubation period it takes at least five days before we can detect an infection.

Typically, what will be done to an animal used in your project?

All birds will have a blood sample taken, along with measurements of wing and weight and fitting of a ring if one is not already present at capture. The entire procedure including standard processing typically takes less than 5 minutes and is undertaken in the field, with birds released back into the wild after it is complete. We would plan to collect up to 1250 blood samples over the course of the project. Approximately 45% of individuals will have a sample taken more than once, and a very small number of individuals (<5) may be sampled in this way on up to five occasions over the course of the project, once per year.

What are the expected impacts and/or adverse effects for the animals during your project?

There may be a small amount of pain during blood collection, but this is likely to be transient. Birds may show panting, gaping, closing eyes, fluffing up feathers or immobility. Birds usually return to normal behaviour (feeding nestlings) within a few minutes of release.

Expected severity categories and the 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 very mild for all animals.

What will happen to animals at the end of this project?

- Set free
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This is a study of the prevalence of a disease in a wild population of a migratory species, and there are no appropriate alternative models available.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to consider that can address our questions.

Why were they not suitable?

Our study is focused on quantifying the distribution of blood parasite vectors across a large part of the globe (Europe and western Africa) and discovering where infection to individual birds occurs, and how often during their lifetime. There is no non-animal alternative model able to do this.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will sample up to 250 individuals per year, which comprises most of the breeding population of our study areas. Such complete sampling across the population is required to encompass the full variation in demography, phenotype and fitness. For example, it ensures we sample those that breed or migrate early/average/late, and individuals of different age and sex. It ensures we sample individuals with previous breeding histories that are already known to us, and that we sample individuals with unknown breeding histories (new recruits to the breeding population) that will be recaptured in later years of the study. Our study species is naturally short-lived, typically just 1-3 years, and so to be able to sample enough individuals that are >3 years old a much greater number of young individuals need to be sampled as natural mortality means this cohort gets greatly reduced (by about 50%) each year. Our sample comprises both males and females, which is important for the study because they have differences in migration timings and African wintering areas that may influence our results. Also there is considerable annual variation in many factors likely to influence patterns in avian malaria across the annual cycle, therefore we need to sample individuals over five years to account for this.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

There are other populations we could additionally sample, but the populations we have selected provide the highest quality data in terms of historical data, population size and recapture rate. Although this work is not of an experimental design, the sample sizes need to provide enough power to handle modelling with multiple explanatory variables (e.g. sex, age, timing, body condition etc) in addition to those of interest (infection rate).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Sample sizes will be larger in the early years of the project. In later years it should be possible to reduce numbers by targeting individuals for sampling for the parts of the analysis that require older birds. However, we will still need to sample some other individuals in later years to account for the effect of between year variation. Any remaining samples following analysis are shared with other research groups to maximise the information gained from the animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our study model is the pied flycatcher, which is a well studied model system for large-scale research including studies of their parasites. They make an ideal system because they breed at high enough densities, in nestboxes, to enable sufficient sample sizes to be obtained and are very resilient to interference. As such an ideal model system a network of similar well studied populations exists throughout Europe, which enables collaborative work such as this to take place.

The method we will use to collect blood samples is a standard and well used method and is the least invasive way of identifying blood parasites. Alternative methods (such as the collection of muscle tissue) would be more painful, stressful and more likely now to cause lasting harm to the animals.

Why can’t you use animals that are less sentient?

Our questions are focussed on the prevalence of blood parasites in adult birds in the wild. It is not possible to test this in immature life stages because immature life stages are very short and so they can rarely get infected, and if they do get infected the 5 day incubation period of malaria means the window for detection is too small, as they leave the nest at 16 days old. Likewise, less sentient models or terminally anaesthetised animals would not enable us to address our questions of interest.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The effect of the procedure is likely to be transient and is a well-established procedure. However, all animals are marked offering the opportunity to assess the behaviour and fitness of a subset of individuals post release.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to consult the species/taxon focussed literature and the relevant Home Office wildlife research pages for new approaches and guidance. Likewise, there will be ongoing discussions about approaches with colleagues, veterinary teams, technicians, the HO inspector and the BTO for ongoing advice. We also follow the ASAB Guidelines for the treatment of animals in behavioural research and teaching (https://doi.org/10.1016/j.anbehav.2019.11.002).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay current with the literature on advances in the methodological approaches used in the project and adapt any paradigms as feasible. In particular we will work closely with our NACWO and NVS to ensure that we are up-to-date with developments in other research groups within our institution, as well as in our collaborating institutions across Europe.

Home Office

103. Genes and mechanisms involved in genome regulation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stem cells, Chromatin, Mitochondria, Brain development, Neurodegeneration

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 study how genes affect the nuclear and mitochondrial genome in stem cells and their progeny

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 DNA in our cells (also known as our genome) is separated in two different places within our cells: the nuclear genome is found in the nucleus, and the mitochondrial genome resides in the energy- producing parts of our cells called the mitochondria. The main function of many of our genes is to organise these genomes, control their replication, repair damage and regulate the expression of other genes.

In this project, we plan to study how genes affect replication, stability and transcription of the DNA (from either genome) in neural stem cells and their progeny (nerve cells and glial cells) in the brain. Many neurodevelopmental and neurodegenerative disorders are

caused by changes in gene expression and by the accumulation of mutations in the nuclear and mitochondrial genomes. However, it is not always known which genes are responsible for these changes. We aim to uncover how changes in our genomes arise, by studying how disease genes as well as novel genes with unknown functions may impact on genome regulation and expression, both in the nucleus and the mitochondria, during normal development and aging of the brain.

By studying how genomes are regulated in normal circumstances, we can learn about what might go wrong, for example in cancer and neurodegenerative disorders. In addition, studying these processes in cells and animals with specific mutations involved in developmental and neurodegenerative diseases will advance our understanding of pathology in humans and eventually lead to novel therapies.

What outputs do you think you will see at the end of this project?

The main output of this project will be an advanced understanding of the processes involved in development and aging of the brain, both in healthy conditions as well as in disease. A first part of the project will lead to a better understanding of whether and how genes that are known to be involved in brain development, aging and neurodegenerative diseases, impact on regulation of gene activity in the brain. In a second part we will identify novel genes and mechanisms that specifically regulate genes in the mitochondria, the energy factories of our cells. This second part will provide new hypotheses and research avenues for future research projects about mitochondrial and neurodegenerative diseases.

The results from our research will be communicated primarily through publications in open-access peer-reviewed journals, with details of the research methods according to the ARRIVE guidelines. These publications will be archived in Europe PubMed Central. When appropriate, we will deposit papers to open-access preprint servers like BioRxiv upon initial submission. Together, this will ensure early and broad dissemination of the data, but at the same time provide rigorous peer-review to guarantee the quality of the results and the interpretations. All forms of raw data will be made fully accessible with unrestricted access once published. This will provide other researchers the opportunity to benefit from the data, and to reproduce results and interpretations. Data generated from this project will form the basis for new funding applications, and may eventually inform future clinical trials.

Throughout the research, novel tools will be developed. These will also be described in open-access peer-reviewed journals and be made fully accessible to other researchers, either by distributing them upon request, or by depositing tools and protocols in public repositories. When appropriate, intellectual property may lead to patent applications.

In addition to scientific publications, we will engage directly with other researchers through participation in meetings, which provide an opportunity to share non-published results at an early stage during the project. As a clinician-scientist, it is important to me that clinicians and patients remain aware of progress made through basic research. For the general public, output will be disseminated and discussed through active public engagement and outreach activities.

Who or what will benefit from these outputs, and how?

This proposal constitutes mainly fundamental research into a biomedical question about normal development and functioning of our developing and aging brain.

The short-term impact of this research will mostly be for academic and clinical researchers with an interest in brain development and regulation of the nuclear and mitochondrial genomes. The molecular tools we have previously developed, the further improvements we plan to make, and the approach we use by first verifying our tools and hypotheses in non-regulated model systems (e.g. in fruit flies or in cell culture) before translating our findings into the mammalian brain, will benefit a wide range of researchers. The findings and tools we generate will be freely shared with other researchers through open-access publications, depositing data in public repositories and presentations at scientific meetings.

We anticipate that on the longer term, this research will benefit patients and their family members, primarily those with neurodevelopmental and inherited neurodegenerative and mitochondrial diseases. Once published, our data will be available to pharmaceutical companies who may use the results to guide development of new treatments. When obvious targets emerge from our research, we will actively pursue these further and explore possibilities to collaborate with expert labs and pharmaceutical industry to maximise the potential therapeutic impact of the findings.

How will you look to maximise the outputs of this work?

Apart from the novel biological insight, a major anticipated benefit from our previous work was related to development of novel molecular tools that could open up research opportunities in other fields. The molecular tools are freely available from the lab, and already prior to publication of our tools, we have set up several collaborations with groups in other research fields to apply our tools in mammalian cell culture systems and other organisms.

This open sharing approach will continue to be a major feature of our future research projects and culture, and will allow us to maximise the outputs of our work. The environment where this research project will primarily be conducted is highly interactive and fosters collaborations. We aim to share non-published results at an early stage during the project, through oral and poster presentations at conferences, and regular informal interactions with other scientists within and outside our institute where we can discuss scientific progress, preliminary data and share ideas, tools and reagents.

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.

Most of the research carried out in mice will be based on data obtained from studies in the brain of fruit flies (Drosophila melanogaster). In a first part of the project, known disease genes and homologs of factors that we previously showed to play a role in development of the Drosophila brain will be tested for their role during mammalian brain development and aging.
Development of the outermost layer of the brain (the cerebral cortex) in the mouse embryo

is the most widely used model system of mammalian (and human) neurogenesis. Embryos between 11 and 14 days (E11-E14) after conception are mostly used, because these stages precede and overlap with the peak of neurogenesis in the mouse cerebral cortex, which is the biological process we are interested in. In addition, well-established protocols exist that allow targeted genetic manipulation of specific cell types in the brain during these developmental stages, without affecting overall development, wellbeing and survival of the mouse embryos.

Typically, what will be done to an animal used in your project?

This project will rely on the maintenance and breeding of both wild-type and transgenic mice. Transgenic mice have small modifications in their genome that allow us to activate or prevent the expression of specific genes in particular cells of the brain. These modifications do not affect the normal development and physiology of the mice and the mice are essentially healthy. Some of these modifications only become functional (i.e. start or stop to express a specific gene) upon injection of specific substances. Some mice will therefore receive injections with these substances at doses that are otherwise harmless and usually do not cause any side-effects.

Some wild-type or transgenic mice will also undergo a surgical procedure called 'in utero injection and electroporation'. In this procedure, pregnant female mice will be subjected to laparotomy (a surgical procedure that involves opening the abdomen of the mouse). Embryos in the second half of pregnancy will receive injections into the fluid-filled cavities of the brain, through the wall of the uterus which otherwise remains intact. These injections contain DNA or RNA that will modulate the expression of specific genes in a subset of the cells in the brain. After injection, electrodes may be placed onto the uterus, across the brain of the embryos, and small currents delivered through these electrodes allow injected DNA or RNA to enter the stem cells of the brain (a process called 'electroporation').

Alternatively, RNA can be embedded in a virus that will enter the stem cells of the brain without prior electroporation. The total procedure lasts about 30 minutes (about 5 minutes per single embryo), and the majority of embryos and mice are expected to recover uneventfully. In a minority of the embryos, the injection may cause damage, either to the brain or to the amniotic sacs that wrap around the embryo.
This will lead to death and resorption of the embryo by the mother.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals that are routinely bred in the context of this project are not expected to exhibit any harmful phenotype or show signs of adverse effects that impact materially on their general well- being. Injection of substances is also not expected to cause harm or adverse effects. Of those animals that will undergo surgical procedures, the majority of the animals are expected to recover uneventfully from surgery. Distress before, during and after the surgery will be kept to a minimum by good surgical practice and the use of pain relief medication. Animals exhibiting any unexpected harmful phenotypes 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)?

Mice, mild, 85%.

Mice, moderate, 15%.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although there are currently no reliable methods that allow us to answer our key questions without use of animals, we have previously fully exploited other possible resources, and will continue doing so in the future. In the past, we have been heavily involved in the development of both invertebrate and non- animal model systems that strikingly recapitulate many events that occur during mammalian brain development and that improve our understanding of nuclear and mitochondrial genome regulation.
Moreover, we regularly collaborate with research groups both within and outside the institute that are experienced in embryonic stem cell-derived models of brain development. Nevertheless, validation of these findings in the context of a living animal, either in genetically modified mice or through surgery on mouse embryos during their development in the uterus, remains central to the study of brain development and essential to allow future clinical translation.

Which non-animal alternatives did you consider for use in this project?

Most of the research carried out in mice will be based on data obtained from studies in fruit flies. In addition, we heavily rely on cell culture to complement fruit fly research, and to optimise tools and techniques prior to applying them in vivo in mice. We will continue following this approach and will continue to collaborate with research groups both within and outside the institute that are experienced in embryonic stem cell-derived models of brain development and aging.

We are aware of recent developments in human and mouse cerebral organoid models, also as recognised by the NC3Rs. However, the current models still have limitations (cost, required technical expertise, variability, etc) that prevent us from applying this on a routine basis in our lab. Nevertheless, we actively explore these possibilities, which might result in future collaborations to extend the findings from our work, both in mouse, cell culture and Drosophila.

Why were they not suitable?

Non-animal alternatives are highly suitable to meet part of our Objectives. This clear focus on replacement has previously led to substantial reduction in animal usage for our research, and allowed us to answer some of the basic scientific questions we are studying

in other non-regulated model systems. Nevertheless, brain development and aging in fruit flies or even in cells cultured in a dish does not recapitulate everything that occurs in a living human brain. In addition, several aspects of how our nuclear and mitochondrial genomes are regulated are specific to mammals, or can only be studied when cells are in the context of a living animal. Therefore, relevant findings from these other models will need to be confirmed in mice before we can consider translating them to a clinical context.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 routine breeding of transgenic mice and maintenance of mouse colonies, we plan to use 3600 mice (9 transgenic strains, each 2 cages of breeding stocks with 8 animals, replaced every 8 weeks over maximum of 5 years). The number of animals that will undergo surgery will depend on the number of factors that we plan to test over the course of the project, but we envisage to use 600 adult mice. This will allow us to study 20 genes in several embryos per experiment, and conduct at least 10 replicates in 3 different cell types of the brain).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

With the approaches described above, which combine experiments in Drosophila, cell culture and mice, we are able to design the best and most efficient experiments and use only a minimum number of animals, but with the prospect to generate information of the highest quality. Experiments are designed according to the PREPARE guidelines, taking advantage of the experimental design checklist (https://norecopa.no/PREPARE), following NC3Rs guidance (https://nc3rs.org.uk/3rs-advice-project- licence-applicants-reduction and https://www.nc3rs.org.uk/experimental-design-assistant-eda).

In addition, the technique we plan to use (DNA adenine methylation identification, DamID) to determine whether and where proteins associate with the nuclear and mitochondrial genomes is highly sensitive and specific, and has previously been extensively optimised. Its major strength lies in the need for very little biological material, in contrast to many of the other currently employed techniques that generate the same types of data. We are now able to obtain meaningful data about where specific proteins bind to the genomes from single mouse embryos and as little as 10,000 cells. This technical advantage will help to design the best and most efficient experiments and use only a minimum number of animals to allow substantial reduction.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Apart from good experimental design, the major optimisation lies in the prior validation of our experimental approaches and genes of interest in non-regulated model systems. In addition, since many experiments will rely on animals that have not undergone genetic

modifications and we will only use the offspring, we will actively engage with tissue-sharing schemes at our institute.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

When studying development and aging of the brain, mice are currently the most heavily studied and best understood animal that closely resembles human development. In addition, they offer the possibility for genetic manipulation. All animals, and particularly those that have undergone surgery, are monitored to ensure that they receive appropriate care and to minimise suffering.

Why can’t you use animals that are less sentient?

Although a major focus of our research is on the use of less sentient animals (in particular fruit flies), brain development and aging in these flies obviously does not recapitulate everything that occurs in a living human brain. Several aspects of brain development and gene regulation are also specific to mammals, and do not occur in other less sentient animals. Therefore, relevant findings from these other models will need to be confirmed in mice before we can consider translating them to a clinical context.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Optimisation of the surgery procedure (in utero injection and electroporation) has been an important part of our previous work and this will remain the case. We have previously introduced several refinements compared to standard working practices, inspired by the Home Office regulations and extensive discussions with the veterinary surgeon and technical support team. This included optimisation of pain relief during and after surgery; change of anaesthetic agents from long-lasting injected agents to inhaled isoflurane with less side-effects upon recovery; optimisation of sterile technique according to Home Office and LASA guidance; improved post-operative care, bedding and food as suggested by the technical team from the animal facility.

All animals will be monitored to prevent them from being distressed or experiencing harmful phenotypes and adverse effects and their bodyweight will be followed to ensure that humane endpoints are adhered to. Pain will be managed adequately through use of pain relief medication and anaesthesia during and after surgical procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgery will be conducted according to LASA Guiding Principles for Preparing for and

Undertaking Aseptic Surgery. We will take the PREPARE and ARRIVE guidelines into account, and regularly consult the advice and guidelines from the NC3Rs (https://nc3rs.org.uk/3rs-advice-project-licence- applicants-refinement).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly receive and read the information that is distributed by the Named Information Officer. The facility where the animal experiments are conducted has an active training and education programme to ensure that researchers remain updated about advances in 3Rs, and I plan to discuss my research, approaches and techniques regularly with scientists, animal technicians and Named People (in particular Animal Care and Welfare Officers and Veterinary Surgeons) within and outside the institute.
External resources that will be regularly consulted are The National Centre for the 3Rs (https://nc3rs.org.uk/resource-hubs), the Laboratory Animal Science Association (LASA), the Institute of Animal Technology (IAT), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA).
Norecopa (https://norecopa.no/databases-guidelines) provides an up-to-date database and guidelines regarding the 3Rs that will be used as an easily accessible online resource.

104. New Roles of the Rho GTPase Signalling Network in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Rho GTPase signalling, Guanine-nucleotide exchange factors, Bacterial infection, Inflammation, Glucose metabolism

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?

Our aim is to increase our understanding of the molecular and cellular processes that enable a healthy lifespan and that, when deregulated, can cause or worsen diseases such as immuno-deficiencies, chronic inflammation and type-2 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?

A better understanding of the biological mechanisms underlying human health and the causes of complex diseases will allow us to better treat such diseases in the future, by enabling us to use rational strategies in the development of more efficient therapeutics with fewer undesirable side-effects.

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 and that - when deregulated - can cause or exacerbate complex diseases such as immuno-deficiencies, inflammatory and metabolic disorders. 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 and less toxic 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 pharmacological industry may benefit by deciding to develop new therapies on the basis of our findings. Eventually, society as a whole may benefit from the improved health brought by such therapies.

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: 3630 per year

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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, some will be compared to old mice, and a few will be tested throughout their lifespan. We will compare young and old mice because some phenotypes, such as inflammation and metabolic syndrome are age-dependent.

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 ex vivo after the animals are humanely killed. Some mice will be aged, so their inflammatory, immune and metabolic responses can be compared to those of young mice. Some mice will be given single injections to challenge their immune system for observation during a few hours or days, or for altering their blood sugar levels. Some mice will be tested throughout their lifetime by blood sampling, either on a healthy or unhealthy diet, in order to monitor the effects of ageing and diet on their blood sugar levels. A few mice may be used for imaging under anaesthesia, for close monitoring of their inflammatory or immune response.

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 inflammatory response. Few animals may experience a more sustained inflammatory response. Rarely, animals may experience health problems associated with weight gain during ageing caused by access to unlimited food supply. 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)?

Around 60% of the genetically modified mice will experience mild severity at worst from being bred and maintained in our excellent animal facility and are not expected to show any overt phenotypic signs due to their genetic modification. Around 20% of mice will experience mild severity at worst from a mild and transient inflammatory response. Approximately 10% of the mice will experience moderate severity at worst from a more sustained inflammatory response, and round 10% are expected to experience moderate severity at worst associated with weight gain during ageing caused by access to unlimited food supply.

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 the whole body, namely inflammation, the clearance of infections and metabolic homeostasis. 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 isolated primary cells isolated from mice and research involving in vivo experimentation in mice to achieve the aim of our project.

Which non-animal alternatives did you consider for use in this project?

We use cell lines and purified proteins widely and wherever possible to study the Rho GTPase signalling network.

Why were they not suitable?

Tissue culture and purified proteins are very valuable in some aspects of our research. However, there are limits, as the cell types that we are most interested in are terminally differentiated and cannot be cultured, and their protein composition cannot be modified other than by genetic means. Our main cell model, neutrophils, are also very short-lived. Hence, we must resort to primary cells that we isolate freshly from genetically altered mice and compare these with cells from wild type control mice. In addition, isolated cells, whether primary or cultured, cannot give the physiological or pathophysiological context of the complex biological systems we are interested in. To understand this context, in vivo experimentation is the only option.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 group sizes that can confidently detect significant differences, determined largely by past experience, and also from the published literature, with help from the Institute's statistician where needed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We strive for minimal variations in age, sex and genetic background between groups to reduce variability. We use blinding and randomisation of samples where possible to minimise bias. All scientific staff are trained in statistical methods and have support from our statistician to design experiments and analyse results. We cryopreserve mouse strains that are not in current use, in order to reduce numbers of actively breeding strains.

During our previous project licence, we made two particular advances to reduce animal numbers:

The proteins we work on regulate a wide range of physiological and pathophysiological responses. Our research has identified a novel regulator of one of these proteins. We had planned to study the role of this protein in neurons using genetically altered mice. Instead, we generated a genetically altered neuronal cultured cell line by CRISPR knockout, which allowed us to study the role of this protein in vitro, without the need for primary cells. This has eliminated the need for generating and testing the genetically altered mouse strains.

In collaboration with our statistician, we have reduced the number of control animals needed for certain in vivo experiments. This became feasible when new versions of GraphPad software were developed for such purposes, which allow meaningful statistical analysis when the size of the control group is smaller than the test group. For example, in experiments where we know the response to be minimal in the sham-treated groups but high in treated groups, we now routinely use a single mouse in the sham- treated group

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 perform pilot studies for models that are new to us, to determine numbers of mice needed and to optimise conditions. Increased use of in vivo imaging will allow us to obtain richer data sets from each mouse and thus reduce the number of mice used. We will share tissues with other laboratories wherever feasible. We cryopreserve mouse strains that are 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.

Wherever possible, mice will be used for isolation of primary cells and tissues after they were humanely killed, rather than for in vivo experimentation. 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 potential functional impacts 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 evaluate phenotype prior to using relevant in vivo models, starting with mild severity procedures and progressing to moderate severity depending on evidence gathered.

We use bacterial infections to challenge the immune system of mice and evaluate their ability to clear these infections. These infections would cause some of the mice to develop clinical signs and even die, if these mice were kept for several days after the infection. We will limit the period of time that infected animals are kept so that we can detect an inflammatory or immune response, without clinical signs or deaths expected.

Bone marrow transplantation may cause up to 5% of mice to die from failed engraftment. Use of this procedure is justified because it informs on the cell types involved in an inflammatory or immune response, thus minimising the need for additional mouse strains. Treatment of the mice with antibiotics and careful timing and dosing of the transplant material will be used to refine this procedure and minimise harm.

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 biopsy mice from the first litter for genotyping, and we use ear rather than tail biopsies. 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. Mice whose immune system we challenge will be monitored closely for body weight or adverse behaviours. Mice for long term evaluation of blood sugar levels will be habituated to handling, and we use a refined method of blood sampling to reduce stress to these animals. Mice on these long-term studies, and all our breeders, have extra enrichment in their cages to further eliminate stress. 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 local campus guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I periodically check the NC3Rs website which gives excellent advice and advertises upcoming seminars, helping me scan for advances and new ways to implement the 3Rs. In addition, our Home Office and AWERB liaisons keep us informed of 3Rs seminars and events.

105. Elucidating the neuroscience aspect of cancer biology to identify novel therapeutics.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, nerve cells, therapy, metabolism, immunology

Animal types	Life stages
Mice	adult, embryo, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the interactions between tumours and the nervous system, and to develop more efficient cancer treatments by modulating this crosstalk.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is known that malignant cells of various cancers acquire nerve cell-like properties, becoming electrically excitable and expressing proteins typical for nerve cells. In the proposed project, we will explore if the appearance of nerve cell-like properties in cancer cells correlates with the extent of tumour growth in mouse models of lung and pancreatic

cancers. Furthermore, tumours of various origin are often richly innervated by the nervous system, which may support cancer growth. We will also investigate if the nervous system sends signals to tumours to regulate their growth and determine whether tumours themselves “communicate” with the whole body by sending signals via the nervous system. Finally, we will check if stimulation or inhibition of such interactions between tumours and the nervous system affects tumour growth. We hope that answering these intriguing, relatively unexplored questions about the roles of nerves and neuronal features in cancer will lead to the development of novel therapeutic inroads for cancer patients.

What outputs do you think you will see at the end of this project?

In this project, we expect to learn a lot of new facts about how cancerous cells use nerve signalling to maintain tumour growth. The new information will be shared with other researchers and clinicians in the form of peer-reviewed original research articles and reviews in specialised scientific journals as well as, potentially, in cancer biology book chapters. We also aim to develop novel therapeutics for cancer patients, including both drugs and devices.

Who or what will benefit from these outputs, and how?

In the short-term, research scientists and students of cancer biology would benefit from the knowledge we aim to generate in this project. In the long-term, novel therapeutics may be developed based on the new biological mechanisms that we will discover. Therefore, ultimately patients with various types of cancer may benefit from these novel treatments.

How will you look to maximise the outputs of this work?

I will seek to publish these results, attend conferences and give seminars to disseminate new knowledge obtained from the project. If any new therapeutics are successfully developed from the project, I will also collaborate with clinicians and reach out to pharmacological companies for potential clinical trials in human patients.

Species and numbers of animals expected to be used

- Mice: 8,680

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.

This project is focused on modelling the interactions between tumours and the nervous system *in vivo* to identify novel therapeutics for cancer patients. Therefore, we will need to use a model system that is able to establish tumours and has a nervous system closely resembling that of human. On the basis of such criteria, adult mice would be the most appropriate animal model for our project, because they have a shorter latency of tumour development compared to higher vertebrates, yet their physiology, anatomy and pathological manifestations better resemble those in humans compared to the features of

other commonly used animal models, such as nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), or zebrafish (*Danio rerio*).

Typically, what will be done to an animal used in your project?

Most of the animals used in this project will develop primary tumours of the internal organs, such as the lung, pancreas, and mammary gland. These primary tumours may metastasise to other parts of the body, such as the brain or liver, causing formation of secondary tumours, as it happens in human cancer. The tumours will either appear spontaneously due to inherent genetic alterations or be induced by the administration of viruses that will carry enzymes enabling such genetic alterations or by a transplantation of cancerous cells.

After tumour induction, animals will undergo different experimental procedures. Animals may be sampled for small portions of blood to monitor changes in various biochemical parameters related to cancer development. We may treat animals with chemical substances that switch on or off the function of some genes, that could be relevant to tumour growth, or with labelling agents that would facilitate visualisation of certain types of cancer cells or cells interacting with the tumour. Some animals will be irradiated and receive bone marrow transplantation to enable precise modulation of their immune system. Animals may undergo imaging to monitor tumour growth by using both modalities that are used in humans, e.g., ultrasound, MRI or PET, and by state-of-the-art specific animal imaging approaches based on luminescence and fluorescence measurements. In some imaged animals, a transparent window may be implanted to aid visualisation of internal organs. Animals may also undergo surgical implantation of devices that would allow to stimulate the activity of certain cells by using light stimulation. Some complicated experiments, where the activity of nerve cells will be stimulated and recorded in vivo, may be performed on animals under terminal general anaesthesia. Most of the animals as well as control counterparts, which will not have tumours or will not undergo the abovementioned procedures, will be culled by approved humane methods and their tissues will be dissected and used in experiments in vitro to examine the molecular and cellular mechanisms of the processes driving cancer growth.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the experimental animals in our project will develop some forms of tumours. In general, tumours in mice either emerge spontaneously or are triggered by a transplantation of malignant cells. In mice genetically predisposed to develop cancer spontaneously, tumours typically develop in the period between 3 to 6 months; in some cases, up to 12 months. Depending on the site of the tumour development and its stage, animals will experience different adverse effects. Animals with liver tumours may develop anaemia, jaundice and ascites (abnormal build-up of fluid in the abdomen). Mice with pancreatic cancer may have low blood sugar and because of the tumour burden, paralysis of hind paws may occur. Animals with lung cancer may be asymptomatic initially, but after several months may develop breathing difficulties and anaemia. In addition, a proportion of the lung tumours may be highly metastatic and in the end stage, lung cancer may be accompanied by disturbances in other organs affecting animal well-being. In animals with spontaneously emerging (autochthonous) tumours, these side effects typically occur at the end stage or tumour development, gradually developing over 1–2 months.

For transplantation models, the time frame between tumour transplantation to the humane endpoint is shorter, typically between 1 and 2 months. Some of the adverse signs

mentioned above for spontaneous cancer models will therefore develop over a shorter period of 1–2 weeks. In addition, mice undergoing transplantations may be impacted by the effects of injections, which could cause local haemorrhage and damage to the organs being injected. For example, mice injected with tumour cells into the spleen may develop mild bleeding from the spleen.

In all cases, tumour burden will be limited to the minimum required for a valid scientific outcome. Animals may display tumour ulceration, laboured respiration, or persistent diarrhoea, but they will be immediately culled after any of these symptoms is observed. The animals will be also monitored for weight loss and body condition score, and mice dropping below the set criteria (e.g., 15% weight drop) will be immediately culled to prevent excessive suffering.

Animals that will undergo surgical procedures may experience mild to moderate pain immediately after the surgery and they will be given analgesics to minimise post-surgical pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the majority of mice that will be developing tumours in the course of the project or will undergo surgeries (60% in total), the severity will be moderate. For 20% of the animals, which would be, for example, genetically modified but without any obvious clinical signs, or receiving infrequent injections, the severity will be mild. The remaining 20% of animals will be used for tissue collection after being killed by a Schedule 1 method or after a non-recovery procedure under general anaesthesia (e.g., perfusion/fixation).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 crosstalk between tumours and their environment, both locally and systemically, and to formulate novel approaches for cancer treatment based on the modulation of the interaction of cancer cells and nervous system, we need to use animals that develop tumours similar to those in humans.

Which non-animal alternatives did you consider for use in this project?

Some information about the sensitivity of the interactions between cancer cells and nerve cells to different drugs can be gained from experiments in cultured cells. We will therefore use such *in vitro* experiments for more precise planning of the experiments in animals. In

addition, data on the expression levels of different genes and abundance of proteins encoded by them, as well as on concentrations of various biologically active substances and metabolites obtained from cancer tissue samples may provide important clues regarding the role of the nervous system in regulating tumour growth. We will therefore employ computational modelling and bioinformatic analyses of such datasets to identify the most promising drug targets to be validated in experiments *in vivo*.

Why were they not suitable?

These alternatives are complementary to the animal studies, but cannot fully replace the work we are proposing here. In particular, it is not possible to achieve a similar structural and temporal complexity of interactions between tumours and nervous cells in experiments *in vitro*. In cell culture, nerve cells have limited viability and may not grow processes of the same length as *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 numbers were estimated based on our previous experiences, statistical modelling, and predictions for exploratory experiments. For spontaneously occurring tumours (autochthonous models), due to the high variability in tumour progression, each cohort we normally would need around 20 animals per group for preclinical trials, whereas transplantation models would normally need between 10–20 animals per group. We will take preclinical trials in small cell lung cancer (SCLC) model as an example: considering potential treatment combinations, each experiment would normally need at least four groups: control/vehicle treated; standard of care/chemotherapy; new drug; new drug in combination with standard of care/chemotherapy. If the new drug alone does not seem efficacious in pilot experiments, we will only test the combination using the minimal animal numbers possible, and see if we could observe any trend. If not, we will not pursue the full trial. Furthermore, for all novel therapies, the dosing frequencies and drug dosage needs to be optimised first. There are also at least three different trial designs: prevention, intervention and regression, which elucidate the role of a certain manipulation (whether pharmacological, genetic or surgical) at different stages of tumour progression. As such, for each new drug to be thoroughly examined, both in transplantation model and in autochthonous setting, in one trial, we would need at least (20 autochthonous + 10 transplant) × 4 experimental groups × 3 designs =360 animals.

We plan to use mouse models of the following five major cancers: SCLC, lung adenocarcinoma, pancreatic ductal adenocarcinoma, pancreatic neuroendocrine tumour and mammary tumour (breast cancer). Therefore, we would need 360 mice × 5 cancer types = 1,800 animals if we want to run at least one trial per cancer type. We would like to run 10 trials in the course of the project, which will require 3,600 animals. In addition, we will run pilot exploratory experiments with smaller groups of animals, probing different approaches to study the relationship between nervous system and tumours. We will need

approximately twice fewer animals in this initial phase (1,800 in total). Therefore, we will need 3,600 + 1,800 = 5,400 mice in experimental protocols.

Considering that some animals may not develop tumours or may be excluded from the subsequent experiments due to various reasons before overt tumour development, as well as taking into account that some animals will have to be initially generated from breeding of GA mice, where not all progeny may be suitable for experiments, we would need to produce 1.5×5,400 = 8,100 GA animals for the programme of experiments. These 8,100 animals will come partly from the breeding protocol on the current Project Licence (4,860 animals or 60%) and partly from other breeding colonies (3,240 animals or 40%). Finally, we envisage that we may need to generate new genetically altered lines of mice or import some novel genetically altered animals from other laboratories. For this purpose, we will need 580 animals. Therefore, we plan to use 8,100 + 580 = 8,680 mice over the period of 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 consulted statisticians for the sample sizes used in preclinical trials. We will also take advantage of the online tools, including the NC3R Experimental Design Assistant to help us with experimental designs. In addition, we will perform smaller-scale pilot studies: if the preliminary results reveal an interesting trend, we will then perform a new set of properly powered experiments in a separate cohort.

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 first use *in vitro* systems to optimise the experimental conditions and to select for best gene and protein targets to be validated *in vivo*. We will also collaborate with bioinformaticians to mine human patient datasets and identify the most promising genes and pathways to be investigated before planning experiments *in vivo*. We will perform pilot studies for these candidates, and will take advantage of different imaging techniques and sampling methods to obtain multiple data points in one single experiment, thus maximising the output from each cohort of animals. We will work with engineers in our department to develop novel devices to be used in this project, aiming to increase the efficacy of such devices so that we could decrease signal variability and therefore use fewer animals for the experiments. We will also work closely with our histology experts and imaging specialists to obtain higher quality images and videos, so that we can use fewer animals for the imaging analyses. Finally, we will generate tissue banks to ensure that the materials could be utilised for different experiments or even shared with other labs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm

to the animals.

We will use mouse models in this project. Genetic alterations or transplantation methods will be used to induce tumour formation in these animals. These models possess relevant pathological features similar to those in human patients, allowing us to investigate basic tumour biology and to perform preclinical trials. This will obviate the need to use higher vertebrates. Furthermore, whenever possible, we will perform experiments in transplant models, which usually exhibit a faster disease course, thus minimising the duration of distress and lasting harm to the animals. We will only perform key experiments in the autochthonous settings, particularly when we will need to elucidate how tumours develop, evolve, and interact with their native microenvironment. Whenever possible, we will use terminal anaesthesia to perform surgical experiments in animals to decrease their pain and suffering.

Why can't you use animals that are less sentient?

This project is focused on modelling the interactions between tumours and the nervous system *in vivo* to identify novel therapeutics for cancer patients. Therefore, we will need to use a model system that is able to establish tumours and has a nervous system closely resembling that of human. Therefore, mice would be the most appropriate animal model for our project. Less sentient animal models, such as nematodes or flies, will not be suitable for our purpose. Whenever appropriate, we will carry out experiments in terminally anaesthetised animals, but for some of the proposed experiments, we will investigate various methods to intervene with the tumour progression. In such cases, we will need to observe the animals over a prolonged period of time until the assigned endpoints are reached.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

I will work with engineers in our department to develop novel devices to be used in this project, aiming to decrease the discomfort of the animals carrying such devices. I will also consult our NVS to improve the surgical methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our experiments will be planned in accordance with the recently formulated PREPARE guidelines.[1] For surgical procedures, we will follow the guidelines set out in LASA Guiding Principles on Preparing for and Undertaking Aseptic Surgery. With regards to the experiments in cancer models, we will adhere to the Guidelines for the Welfare and Use of Animals in Cancer Research.[2] In addition, I will also keep myself updated with the latest advancements in relevant fields, by attending conferences, reading journal articles, and collaborating with experts in these areas, to ensure that the experiments will be conducted in the most refined way.

Smith, A. J. et al. PREPARE: guidelines for planning animal research and testing. Lab Animals 52, 134–141 (2018).

Workman, P. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep following up the latest publications in the fields and attend courses and seminars to learn any advances in the 3Rs.

106. Regulation of tumour growth and metastasis by altered chemical handling

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cancer, Metastasis, 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 chemicals inside breast tumours are critical for regulating cancer growth and spread. We can manipulate their levels with existing out-of-patent drugs. Combining such treatments with existing approaches represents a novel way to treat breast cancer. The goal of this project is to understand how transmembrane chemical transport contributes to breast cancer progression in order to find new ways to detect and treat patients, improving survival and quality of life at minimal cost.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 the ways that chemical changes help breast cancers to grow and spread will enable effective drugs to be identified. By targeting them in combination with standard treatments, outcomes will improve thereby lowering mortality rates.

What outputs do you think you will see at the end of this project?

Outputs from this project will include new understanding on breast cancer growth and spread. These key findings will be reported in scientific publications.

Who or what will benefit from these outputs, and how?

The main potential benefit of this work is that transport-blocking drugs may be new targets for breast cancer therapy. In addition, a special type of MRI which can detect chemical changes may be a useful new tool for diagnostics. Drugs already in clinical use to treat other diseases such as epilepsy might also be effective for treating breast cancer . By better understanding the role of chemical transport in regulating cancer growth and spread, we should be able to design new, better treatments to improve patient outcomes.

How will you look to maximise the outputs of this work?

Outputs from this work will be maximised through publication of data (including unsuccessful outcomes). Knowledge will be disseminated through collaboration with other researchers (breast cancer researchers, physicists, chemists, radiologists) and clinicians.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our laboratory uses in vitro models as investigative tools whenever possible, and a large part of this work uses cell and tissue culture techniques. However, at this point it is necessary to use mice to answer questions about chemical function during cancer growth and spread in vivo. Cancer spread (metastasis) is a complex process that cannot be accurately mimicked using cell lines or computer models alone. Cancer studies in mice are essential to understand metastatic breast cancer. Mice are unique in their ability to accept, develop and accurately model human breast cancers.

Typically, what will be done to an animal used in your project?

These models involve injecting cancer cells into the breast of female mice. The tumours may grow to a size big enough to cause some discomfort, in which case we will give the mice pain-relieving medication. The tumour, metastases and the response to treatment, can then be seen using specialised imaging systems and microscopes. Some of the mice will be given a non-toxic dose of a drug with or without other chemotherapy. Some of the mice used in this project are immunodeficient so that they can accept human tumours. They will be kept in individually ventilated cages to avoid infections.

What are the expected impacts and/or adverse effects for the animals during your project?

The symptoms experienced by the mice in this project will be of no more than moderate severity. A number of the mice used in this project will develop breast cancer. The tumours may grow to a size big enough to cause minor discomfort, in which case we will give the

mice pain-relieving medication. Mice will be put to sleep at the end of the experiment and their tissues will be collected for analysis and to reduce the need to use further animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% -- moderate

50% -- mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At this point it is necessary to use animals to answer mechanistic questions about tumour growth and metastasis. Metastasis is a complex, integrative process that cannot be accurately mimicked using cell lines or computer models alone. Cancer studies in mice yield valuable insights into our understanding of metastatic breast cancer.

Which non-animal alternatives did you consider for use in this project?

Our laboratory uses in vitro models as investigative tools whenever possible, and a large part of this work uses cell and tissue culture techniques.

Why were they not suitable?

Metastasis is a complex, integrative process that cannot be accurately mimicked using cell lines or computer models alone.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 of animals has been estimated based on similarly designed experiments conducted previously.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have researched and kept up to date with the most refined methods via online resources at NC3Rs (https://www.nc3rs.org.uk/our-resources), relevant methodological publications in the literature, as well as frequent discussions on best practice with other colleagues working with mice. We are fully committed to publishing in accordance 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 use in vitro models and clinical datasets wherever possible in order to limit the number of animals used for this research. We use state-of-the-art imaging approaches, which enables us to generate large amounts of data on tumour growth and metastasis from individual mice, reducing the numbers needed. Finally, we collect and store tissue from the animals used in this project and share these samples with the breast cancer research community in order to reduce the number of animals used in breast cancer research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are used because they are unique in their ability to accept, develop and accurately model breast cancers. The tumour implantation and in vivo imaging will be performed under anaesthesia to minimise suffering.

Why can’t you use animals that are less sentient?

Mice are the easiest mammals to handle and house properly, have a short gestation time (19 days) and relatively short life span (1-3 years). This makes it possible to follow the progress of the experiment in a reasonable time frame.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The tumours may grow to a size big enough to cause some discomfort, in which case we will give the mice pain-relieving medication. Animals will also be monitored using body condition scoring, to be evaluated during the project and refined as necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA: http://www.lasa.co.uk/PDF/AWERB_Guiding_Principles_2015_final.pdf ARRIVE:

https://nc3rs.org.uk/revision-arrive-guidelines
Workman et al guidelines for the welfare and use of animals in cancer research: https://www.nature.com/articles/6605642

LASA re routes of administration https://www.lasa.co.uk/PDF/LASA-NC3RsDoseLevelSelection.pdf

PREPARE guidelines: https://norecopa.no/PREPARE

https://nc3rs.org.uk/3rs-advice-project-licence-applicants-refinement

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with the most refined methods via online resources at NC3Rs (https://www.nc3rs.org.uk/our-resources), relevant methodological publications in the literature, as well as frequent discussions on best practice with other colleagues working with mice. Our institute holds Users Forum meetings twice a year for updates and support for the 3Rs.

Home Office

107. Pancreatic Cancer: Biology and Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pancreatic Cancer, Molecular Pathogenesis, Genetics, Cancer Progression, Therapy

Animal types	Life stages
Mice	embryo, pregnant, adult, 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 goal of this project is to identify and characterise the genetic events and molecular pathways involved in the initiation and progression of pancreatic cancer, and to use this information to discover effective treatments and improved 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?

The five-year survival rate for pancreatic cancer patients is the lowest among common cancers, and remains at only 6%. This unfavourable outcome is related with our deficient knowledge of the molecular pathogenesis of this disease, which has contributed to our inability to intervene more effectively.

Therefore, there is an urgent need to better understand the molecular basis of pancreatic

cancer progression. This will be essential for identifying prognostic and predictive biomarkers, to develop novel therapeutic strategies, and to identify the molecular pathways that should be targeted to benefit the greatest number of patients.

What outputs do you think you will see at the end of this project?

1.- This project will provide novel insights into genetic and cellular events involved in the initiation and progression of this disease from a preinvasive and potentially curable phase to an invasive and lethal stage.

2.- This project may also lead to the discovery of new strategies for treating pancreatic cancer, which is among the most lethal human cancers.

3.- This project will lead to publication of peer reviewed papers.

Who or what will benefit from these outputs, and how?

1.- This research will directly benefit those in the field of pancreatic cancer, including basic scientific researchers and clinicians (gastroenterologists, oncologists, pathologists). Pancreatic cancer is the fourth most common cause of cancer death in western society, with most patients dying within one year of diagnosis. Therefore, there is an urgent need to better understand the molecular basis of pancreatic cancer progression as well as molecular targets that can lead to the development of new therapeutic approaches to tackle this disease. This group will realise the benefit from the research within the timescale of the research and subsequently.

2.- Researchers working on the project. The research staff working on the project will benefit from exposure to multidisciplinary research which will increase their scientific background. This group will realise the benefit from the research within the timescale of the research and subsequently.

3.- Moreover, this research will benefit those working in cancer biology. Our project will develop bridges between research on pancreatic cancer and other cancer types. We expect that the completion of this project will provide the opportunity to understand different aspects of pancreatic cancer progression that can be used in other epithelial neoplasias. This group will realise the benefit from the research within the timescale of the research and subsequently.

4.- This research will also benefit those developing strategies to fight pancreatic cancer. It is expected that this project will reveal new molecular targets to fight pancreatic cancer, leading to more rational selection of strategies for anti-cancer drug development. This group will realise the benefit from the research within the timescale of the research and subsequently.

How will you look to maximise the outputs of this work?

1.- Publication of our research in peer reviewed papers.

2.- Presentations at national and international meetings, and conferences.

3.- We interact with scientists interested in the pancreatic cancer at a local, national and international level and will engage with them in explaining the significance of the findings.

This will allow dissemination of our findings.

Species and numbers of animals expected to be used

- Mice: 1,375

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Genetically modified mice permit specific genes to be knocked out, or removed, at specific time-points in the animal's life appropriate to the development of cancer. This removal can also be localised to a specific organ, e.g. pancreas or lung. Mice are one of the few vertebrate species where gene knock-out experiments can be carried out. Mice also breed quickly allowing inter-crossing in a reasonable period of time. Mice have a short lifespan enabling the study of genetic manipulations from birth through to old age. Crucially, they recapitulate well human cancer behaviour. There is no other model system that is capable of providing the type of data necessary for our studies.

Analysis of pancreatic cancer progression will involve the use of adult mice, and scientific endpoints will include the analysis of both early and late stages of pancreatic cancer development. To study early stages of pancreatic cancer development, pancreata will be obtained and analysed before mice develop pancreatic cancer. To study late stages of pancreatic cancer development, pancreata and other tissues will be obtained and analysed after the development of clinical signs associated with pancreatic cancer. Wild-type animals could be used as control cohorts in these experiments.

Transplantation models involve the use of adult animals (wild-type, genetically modified and immunodeficient mice), allowing a reduction in the number of mice needed and will be employed when they can substitute genetically modified mice.

Cell/Tissue may be collected from embryos (wild-type, genetically modified mice), providing a partial replacement for the use of adult animals.

Typically, what will be done to an animal used in your project?

1.- Breeding and maintenance of genetically altered animals not expressing oncogenic Kras: Mice are usually bred when they are 10-16 weeks-old. These mice are killed at 6-8 month of age, once the next generation is established.

2.- Breeding and maintenance of genetically altered animals expressing oncogenic Kras in the pancreas: Mice are usually bred when they are 10-16 weeks-old. These mice are killed at 6-8 month of age, once the next generation is established.

3.- Tumour evaluation: Some studies will aim to analyse early stages of pancreatic cancer development. Mice included in these studies will be killed before the development of pancreatic cancer (usually, 4-12 months-old mice expressing the oncogene Kras in the pancreas), and they are not expected to show any sign of disease, although they could develop benign papillomas that will not interfere with the study. Other studies will aim to

analyse late stages of pancreatic cancer development. To study late stages of pancreatic cancer development, pancreata and other tissues will be obtained and analysed after the development of external signs associated with pancreatic cancer (usually ≥12 months-old mice expressing the oncogene Kras in the pancreas or 4-6 months-old mice expressing the oncogene Kras and concomitant loss of tumour suppressor genes (eg p53, p16, Smad4) in the pancreas).

4.- Administration of agents for therapy, and analysis: Mice included in these studies will be treated with therapeutic agents. In chemoprevention studies, administration of therapeutic agents will start before the development of pancreatic cancer (usually, ~4 months-old mice expressing the oncogene Kras in the pancreas, or ~8 weeks-old mice expressing the oncogene Kras and concomitant loss of tumour suppressor genes (eg p53, p16, Smad4) in the pancreas). Agents will be administered until the development of clinical signs of pancreatic cancer (usually ≥12 months-old mice expressing the oncogene Kras in the pancreas or 4-6 months-old mice expressing the oncogene Kras and concomitant loss of tumour suppressor genes (eg p53, p16, Smad4) in the pancreas). Other studies will aim to analyse late stages of pancreatic cancer development. Here, therapeutic agents will be administered in mice harbouring pancreatic tumours (usually, ~3-3.5 months-old mice expressing the oncogene Kras and concomitant loss of tumour suppressor genes (eg p53, p16, Smad4) in the pancreas). Agents will be administered until the development of clinical signs of pancreatic cancer (these mice have a median survival of ~5-5.5 months). Some mice could develop benign papillomas that will not interfere with the study.

5.- Grafting of cells or tissue fragments. Transplantation models may be used as alternative mouse models to study pancreatic cancer. Cells/tissue fragments will be administered one time in adult animals.

a.- Administration of cells/tissue fragments subcutaneously (xenograft) or directly into the pancreas (orthotopic, surgical procedure) in adult animals to study biology and/or therapeutic responses. We will use cells and tissue fragments from both human and murine origin.

b.- Metastasis models generated by administration of cells via the tail vein, peritoneum or into the spleen to study biology and/or therapeutic responses. We will use cells from both human and murine origin. Tail vein administration of cells leads to metastasis mainly in lungs, while peritoneum leads to peritoneal metastasis, and intra-splenic injection leads to hepatic metastasis.

6.- Cancer induction in lung tissue, and analysis. This study will involve the use of genetically modified mice. Lung cancer will be induced in adult mice through intranasal inhalation of viruses to induce or delete gene expression. Some studies will aim to analyse early stages of lung cancer development.
Mice included in these studies will be killed before the development of lung cancer, and they are not expected to show any sign of disease. Other studies will aim to analyse late stages of lung cancer development. To study late stages of lung cancer development, lung and other tissues will be obtained and analysed after the development of external signs associated with lung cancer.

What are the expected impacts and/or adverse effects for the animals during your project?

1.- Breeding and maintenance of genetically altered animals not expressing oncogenic

Kras: Animals produced under this protocol are not expected to exhibit any harmful phenotype. However, it is not possible to fully predict the nature or severity of any potential defect and all types of mice will be monitored for possible side effects. Animals exhibiting any unexpected phenotype likely to exceed a mild severity will be killed, or transferred for continued use in protocol 3 (Tumour evaluation).

2.- Breeding and maintenance of genetically altered animals expressing oncogenic Kras in pancreas: Some of the mice produced under this protocol (<25%) are likely to develop benign papillomas (if oncogenic Kras is expressed in the skin due to unexpected expression of the Cre recombinase). Mice that develop papillomas will be killed or transferred for continued use in protocol 3 (Tumour evaluation).

Additionally, some of the mice in this protocol could develop:

a.- Pancreatic cancer. Some of the mice used in this protocol will express oncogenic Kras in the pancreas (<25%). However, these mice are not expected to develop pancreatic cancer within 1 year of life, and mice older than 6 months are not usually used for breeding. Therefore, it is highly improbable that mice in this protocol develop pancreatic cancer (<5% of mice expressing oncogenic Kras in the pancreas develop pancreatic cancer within 6 months of age). Mice that develop pancreatic tumours will be killed or transferred for continued use in protocol 3 (Tumour evaluation).

b.- Other internal tumours. Some of the mice used in this protocol could spontaneously lose a copy of a tumour suppressor genes (e.g. p53) and develop tumours (e.g. lymphomas or sarcomas). This is unlikely, but in this scenario, mice will be killed.

It is not possible to fully predict the nature or severity of any potential defect and all types of mice will be monitored daily for possible side effects. Animals exhibiting any unexpected phenotype likely to exceed a moderate severity will be killed. However, should the development of more harmful phenotypes be necessary to allow maintenance of certain lines of scientific interest, animals will not be allowed to
exceed the limits set in this protocol. Where the immune status of the animals might compromise health, they will be maintained in a barrier environment.

3.- Tumour evaluation: Mice produced under this protocol are likely to develop benign papillomas (≥50%) (if oncogenic Kras is expressed in the skin due to non-target expression of the Cre recombinase in the skin), and/or pancreatic cancer, the impact of which will be assessed as described below.

Some studies will aim to analyse early stages of pancreatic cancer development. Mice include in these studies will be killed before the development of pancreatic cancer, and they are not expected to show any sign of disease, although they could develop benign papillomas that will not interfere with the study. These mice are expected to be killed at 4-12 months of age.

Other studies will aim to analyse late stages of pancreatic cancer development. To study late stages of pancreatic cancer development, pancreata and other tissues will be obtained and analysed after the development of external signs associated with pancreatic cancer. The median survival of these mice range 3-6 months (depending on the genotype).

It is not possible to fully predict the nature or severity of any potential defect and all types

of mice will be monitored daily for possible side effects. Animals exhibiting any unexpected phenotype likely to exceed a moderate severity will be killed. Where the immune status of the animals might compromise health, they will be maintained in a barrier environment.

A small percentage of mice (<10%) may develop other type of tumours (liver cancer, lung cancer, lymphoma, sarcomas) due to leakiness of the Cre recombinase allele, or spontaneous loss of tumour suppressor genes. These mice will be killed.

4.- Administration of agents for therapy, and analysis: The majority of mice on this protocol are likely to develop tumours, the impact of which will be assessed as described below.

Some mice are likely to develop benign papillomas (≥50%) (if oncogenic Kras is expressed in the skin due to non-target expression of the Cre recombinase in the skin), and/or pancreatic cancer, the impact of which will be assessed as described below.

In chemoprevention studies, administration of therapeutic agents will start before the development of pancreatic cancer (usually, ~4 months-old mice expressing the oncogene Kras in the pancreas, or ~8 weeks-old mice expressing the oncogene Kras and concomitant loss of tumour suppressor genes (eg p53, p16, Smad4) in the pancreas). Agents will be administered until the development of clinical signs of pancreatic cancer (usually ≥12 months-old mice expressing the oncogene Kras in the pancreas or 4- 6 months-old mice expressing the oncogene Kras and concomitant loss of tumour suppressor genes (eg p53, p16, Smad4) in the pancreas). Other studies will aim to analyse late stages of pancreatic cancer development. Here, therapeutic agents will be administered in mice harbouring pancreatic tumours (usually, ~3-3.5 months-old mice expressing the oncogene Kras and concomitant loss of tumours suppressor genes (eg p53, p16, Smad4) in the pancreas). Agents will be administered until the development of clinical signs of pancreatic cancer (these mice have a median survival of 5-5.5 months). Some mice could develop benign papillomas that will not interfere with the study.

It is not possible to fully predict the nature or severity of any potential defect and all types of mice will be monitored daily for possible side effects. Animals exhibiting any unexpected phenotype likely to exceed a moderate severity will be killed. Where the immune status of the animals might compromise health, they will be maintained in a barrier environment.

A small percentage of mice (<10%) may develop other type of tumours (liver cancer, lung cancer, lymphoma, sarcomas) due to leakiness of the Cre recombinase allele, or spontaneous loss of tumour suppressor genes. These mice will be killed.

5.- Grafting of cells or tissue fragments: The majority of mice will develop tumours (close to 100%) when grafted with tumour-derived cells. Different studies will be performed:

a- Tumour models generated by the administration of cells or tissue fragments subcutaneously (xenograft). We will use cells and tissue fragments from both human and murine origin. These mice will develop external tumours, and will be killed when the estimated external tumour weight is 10% of the body weight, or when the tumours produce skin ulcerations. It’s very unlikely that these mice develop internal tumours or additional adverse effect. In this scenario, mice will be killed. Mice will be killed usually ≤10 weeks after cell transplantation, or ≤6 months after tissue transplantation.

b.- Tumour models generated by the administration of cells or tissue fragments into the pancreas (orthotopic). We will use cells and tissue fragments from both human and murine

origin. These mice will develop pancreatic cancer. Some studies will aim to analyse early stages of pancreatic cancer development. Mice included in these studies will be killed before the development of pancreatic cancer, and they are not expected to show any sign of disease. Other studies will aim to analyse late stages of pancreatic cancer development, and pancreata and other tissues will be obtained and analysed after the development of external signs associated with pancreatic cancer. Mice will be killed usually ≤6 months after cell/tissue transplantation.

c.- Metastasis models generated by administration of cells via the tail vein, peritoneum or into the spleen. We will use cells from both human and murine origin. Tail vein administration of cells leads to metastasis mainly in lungs, while peritoneum leads to peritoneal metastasis, and intra-splenic injection leads to hepatic metastasis. These studies will not require the development of advanced disease and mice will be killed usually ≤8 weeks after cell transplantation. Imaging approaches (optical imaging) will be considered to define earlier endpoints to terminate the procedure before mice start to show external signs of disease.

It is not possible to fully predict the nature or severity of any potential defect and all types of mice will be monitored daily for possible side effects. Animals exhibiting any unexpected phenotype likely to exceed a moderate severity will be killed. Where the immune status of the animals might compromise health, they will be maintained in a barrier environment.

A small percentage of mice (<10%) may develop other type of tumours (liver cancer, lung cancer, lymphoma, sarcomas) due to leakiness of the Cre recombinase allele, or spontaneous loss of tumour suppressor genes. These mice will be killed.

6.- Cancer induction in lung tissue, and analysis: Mice produced under this protocol are likely to develop lung cancer (≥50%, if oncogenic Kras is expressed in the lung), the impact of which will be assessed as described below. Lung cancer will be induced through intranasal inhalation of viruses expressing recombinases to induce expression of oncogenic Kras in 6-10 weeks old mice, and mice will be usually killed ≤6 months after Kras activation.

Some studies will aim to analyse early stages of lung cancer development. Mice include in these studies will be killed before the development of lung cancer, and they are not expected to show any sign of disease.

Other studies will aim to analyse late stages of lung cancer development. To study late stages of lung cancer development, lung and other tissues will be obtained and analysed after the development of external signs associated to lung cancer.

It is not possible to fully predict the nature or severity of any potential defect and all types of mice will be monitored daily for possible side effects. Animals exhibiting any unexpected phenotype likely to exceed a moderate severity will be killed. Where the immune status of the animals might compromise health, they will be maintained in a barrier environment.

A small percentage of mice (<10%) may develop other type of tumours (lymphoma, sarcomas) due to spontaneous loss of tumour suppressor genes. These mice will be killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

1.- Breeding and maintenance of genetically altered animals not expressing oncogenic Kras: 100% of mice (MILD).

2.- Breeding and maintenance of genetically altered animals: 75% of mice (MILD) and 25% of mice (MODERATE).

3.- Tumour evaluation: 50% of mice (MILD) and 50% of mice (MODERATE).

4.- Administration of agents for therapy, and analysis: 100% of mice (MODERATE).

5.- Grafting of cells or tissue fragments: 100% of mice (MODERATE).

6.- Cancer induction in lung tissue, and analysis: 50% of mice (MILD) and 50% of mice (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?

Wherever possible our laboratory carries out experiments on cultured cells. However, the interactions of neoplastic cells with other cells within a tissue are too complex to recapitulate in a culture dish. For this reason, it is essential to perform some experiments on animals. However, results from cell culture experiments will be used to design our animal work. Thus, in-vitro work will allow the selection of relevant molecular targets that warrant the use of mouse models to confirm their contribution to pancreatic cancer development.

Mice represent the ideal model organism available, because of the ability to manipulate gene expression and study the tumour within the context of a whole organism. Cancer is a disease of fully formed adult organisms, so the use of early developmental stages of protected animal species, before the regulations apply to them, is also not appropriate.

Which non-animal alternatives did you consider for use in this project?

We use pancreatic cell lines (2D) and 3D culture of organoids to replace the use of animals in our research.

Why were they not suitable?

In-vitro models are valuable platforms to understand the biology of pancreatic cancer, and we employ them in our research to replace the use of animals. However, they cannot

recapitulate the complex interaction between the neoplastic cells and the tumour microenvironment within the context of a whole organism. Therefore, some experiments need to be performed 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?

Experiments are designed in consultation with our in-house statistician in order to use the minimum number of animals. Our work with these mouse models during the last 15 years has allowed us to refine the scientific endpoints to ensure the use of the minimum number of animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The design of the experiments include consultation with a statistician and the analysis of the scientific aims and scientific endpoints that we have gained working with these mouse models during the last 15 years. A typical study will involve cohorts of approximately twenty to thirty tumour model mice and appropriate controls. The therapeutic study usually involves a short-term efficacy trial using approximately ten mice in each group (test and controls). If the therapy impacts some parameter of tumour biology, expanded studies to allow a statistical evaluation of efficacy will be designed; these usually require up to twenty mice in each cohort. Feedback from the NC3Rs EDA is considered in the design of the experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To reduce the number of mice, we will keep stocks of frozen sperm and embryos so that if a mouse line is not continuously required we can avoid unnecessary breeding. To maximise the data obtained from each animal we will collect samples from multiple body sites whenever possible to prevent duplication for future experiments. When possible, and for the generation of complex compound mutant strains (more than 2 mutant alleles), we will generate breeders with more than one mutation (always that the combination of mutations doesn’t have any harmful effect on the mice) to reduce the number of breedings required to generate the experimental cohort. Finally, the use of imaging approaches will help us to understand the progression of the disease and to design strategies to reduce the number of experimental mice needed for each 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 mouse models we use to address each specific question are chosen to achieve the scientific aims with the minimum of animal suffering. Genetically modified mice permit specific genes to be knocked out, or removed, at specific time-points in the animal's life appropriate to the development of cancer.

This removal can also be localised to a specific organ, e.g. pancreas or lung. Mice involved in the study of early stages of tumourigenesis are not expected to undergo signs of disease. To study late stages of cancer, and in order to minimise animal suffering and to refine the humane endpoints, animals will be closely monitored (at least daily) and any animal displaying external signs of undue distress (including piloerection and hunched posture; inactivity; diarrhoea; dyspn006Fea; tachypnoea; hyperpnoea or neurological signs such as gait disturbance) will be immediately killed. This will apply during normal husbandry and breeding but especially while the animals are undergoing regulated procedures.

Transplantation models involved in the study of early stages of pancreatic cancer are not expected to undergo signs of disease. To study late stages of pancreatic cancer, and in order to minimise animal suffering and to refine the humane endpoints, animals will be closely monitored (several times/day following the cell/tissue implantation to ensure full recovery, and then daily) and any animal displaying external signs of undue distress (including piloerection and hunched posture; inactivity; diarrhoea; dyspnoea; tachypnoea; hyperpnoea or neurological signs such as gait disturbance) will be immediately killed. Imaging approaches may be used in these studies (particularly studies involving models of metastasis) to define earlier scientific endpoints.

For therapeutic studies, we will perform pilot experiments with small numbers of mice to ensure that there are no unexpected adverse effects from the treatment and to ensure that there is sufficient evidence to warrant larger scale studies. Mice will be monitored daily and any animal displaying external signs of undue distress (including piloerection and hunched posture; inactivity; diarrhoea; dyspnoea; tachypnoea; hyperpnoea or neurological signs such as gait disturbance) will be immediately killed. Imaging approaches will be used in mice harbouring tumour bigger than 50 mm3 to define earlier scientific endpoints.

Why can't you use animals that are less sentient?

Mice are one of the few vertebrate species where gene knock-out experiments can be carried out. Mice also breed quickly allowing inter-crossing in a reasonable period of time. Mice have a short lifespan enabling the study of genetic manipulations from birth through to old age. Crucially, they recapitulate well human cancer behaviour. There is no other model system that is capable of providing the type of data necessary for our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined all animal procedures through training and advice to ensure that we can

achieve the scientific aims with the minimum of animal suffering. Benign papillomas, which are not the object of our studies, can occur in some of our mouse models before they develop pancreatic tumours, which are the object of our experiments. Mice will be killed if the tumour has ulceration or if the tumour impedes a vital function (for example, locomotion, vision, mastication, excretion).

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 licence will be undertaken in accordance with the principles set out in the Guidelines for the welfare and use of animals in cancer research: British Journal of Cancer (2010) 102, 1555-1577 (referred to subsequently as the NCRI Guidelines).
Furthermore, surgical procedures will be undertaken adhering to the guidelines described 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?

Research in our lab is funded by NC3Rs and we are informed about advances in the 3Rs through the NC3Rs e-newsletter and RPM. Furthermore, I attend workshops and symposiums organised by NC3Rs.

108. Impact of sensory and electrical activity on neuronal function

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

neuroscience, plasticity, olfaction, dopamine, neurogenesis

Animal types	Life stages
Mice	adult, pregnant, 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?

To investigate how different activity-induced plastic changes in shape and electrical activity impact neurons and neural networks, how they are employed to sustain different behavioural needs, and how they could be harnessed to counteract disease. The project will mainly focus on the olfactory system, the part of the brain that encodes smells, and on a class of neurons called dopaminergic, which are important for sensory processing, movement and motivation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 all living organisms the ability to sense and react to the environment is fundamental to survival. Animals must constantly sample the ever-changing environment via their sensory organs (e.g. eyes, nose, skin, ears). They then need to compute the resulting information in brain cells (neurons) and circuits to generate an appropriate behavioural output. When successful, this process, known as neuronal plasticity, underlies processes such as development, learning, memory, adaptation.

With this work I aim to investigate how brain cells and brain circuits respond to changes in input that they receive. This will enhance our understanding of the fundamental principles of how the brain controls behaviour.

What outputs do you think you will see at the end of this project?

Chiefly, outputs will include generation of new knowledge, which will be timely and freely disseminated as publications in open-access journals, full datasets, and conference presentations.

In the medium to long term, the proposed work on how the brain cells and circuits in the olfactory system change to adapt to incoming odour stimuli promises to uncover novel strategies for treating smell disorders.

Moreover, the part of the project that focuses on brain cells called "dopaminergic" is relevant for disorders such as Parkinson’s disease that involve specific pathology to that cell type, and that is associated with early loss of smell. While the work presented in the project is not directly translatable to the clinic, it will hopefully inform future diagnostic and therapeutic approaches.

Who or what will benefit from these outputs, and how?

The proposed project comprises basic neuroscience research, with primary benefits coming from the generation of new knowledge.

By changing the inputs coming from the environment in a controlled way, and adopting and state-of-the- art technology to interact with brain cells, the proposed project will significantly enhance our knowledge of how the environment shapes brain function.

Given its multidisciplinary nature, the work presented in this proposal will be of great interest to a variety of researchers. These include scientists working on, for instance, different brain areas, brain cell development, the five senses and how they influence brain cell function. Moreover, the outputs will inform translational work done by clinician scientists interested in cell transplantation, Parkinson’s and other dopaminergic and/or neurodegenerative disorders. Indeed, I have already secured two collaborations, one with a mathematician interested in generating computer models of the brain, and one with a neurologist whose lab works on translational neuroscience.

I therefore will do my absolute best to ensure that the resulting raw data (physiology, morphology, and behaviour), analysis and manuscripts will be fully and promptly available.

How will you look to maximise the outputs of this work?

I am a strong supporter of open science. Since I have been actively involved in deciding where to publish, I have opted for open source journals or paid the extra fee to make the

paper immediately accessible.

I am also in favour of data sharing: I have uploaded the entire datasets of my most recent work (raw data and analysis) on online repositories. I also am the proud first author of two papers which include ONLY negative findings.

I plan to continue along this path for all research performed in my own laboratory, to cost open science and data sharing into all grant applications, publish negative findings, and, importantly, share full datasets.

Species and numbers of animals expected to be used

- Mice: 6350

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 is looking at how neurons born during different stages of the animal's life respond to changes in incoming stimuli from the environment. To achieve this goal, the project will use mice of different ages and developmental stages. These include a small number of pregnant dams carrying developing embryos and pups (to investigate the developmental origin of various cell types), and adult mice (to investigate how mature neurons respond to change in incoming stimuli, and how these changes drive animal behaviour).

Mice will be used to: a) take full advantage of the transgenic mouse lines that have been developed in recent years; b) build upon the extensive existing mouse literature covering development, shape, physiology and behaviour.

Typically, what will be done to an animal used in your project?

Only transgenic mice which carry no harmful genetic mutations (for instance, having specific neurons coloured in green or red) will be bred in this project. Each mouse will then be used for only one experiment, lasting, on average, a few days, and never more than 4 months. Experiences may include being exposed to more or less odours, and performing a behavioural smell or motor tasks. A minority of mice will be subjected to a single brain surgery, with post-operative care and monitoring and full recovery necessary before undergoing further testing.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals (92%) will be experiencing a series of mild procedures: transgenic breeding, blocking one nostril, behavioural testing.

Less than 1% of animals (20 in total) will undergo vasectomy to generate and mantain transgenic lines.

5.5% of mice will undergo recovery surgery to label, manipulate or make visibile individual brain cells; we do not expect adverse effects. In the extremely unlikely scenario of surgical complications, animals will be immediately killed with an appropriate painless method.

1.6% of mice will receive a single intraperitoneal injection which will result in degeneration of the cells in the nose and a transient loss of their sense of smell. The mice will regenerate these cells, and they within a month or less they will be able to smell as before the injection. We do not expect adverse effects and we will make sure that food and water are easily accessible, that the mice do not have troubles eating and do not lose weight.

A small number of animals (less than 1%, 30 mice in total) will undergo recovery surgery to inject specific substances which kill a subset of dopaminergic brain cells in the midbrain. They may suffer slight difficulties in moving around, associated with early onset of Parkinson's disease which is brought about by the surgical injection. These are expected to be minimal (imperfect posture, walking and coordination), and not lasting longer than a few weeks. Animals will be closely monitored, and adjustments will be made to make sure that food and water will be always within reach.

Expected severity categories and the 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: 92%
Moderate: 8%

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 proposed research necessitates experiments on animals because, to answer the important questions in this proposal there are currently no possible non-animal-based approaches. On one hand, it is not possible to study how learnign at the behaviour level changes without using a living animal, for example, we cannot make individual cells learn to recognise different odours and behave differently as a result of this learning. On the other hand, in order to understand what drives this learning and behavioural adaptation, we need to investigate how brain cells change their shape and function at the microscopic level, something that we cannot do in a human at the necessary granular level of single current in individual neurons. To link the two levels of understanding - cells and whole organism - and to relate the findings to what happens in the human brain, we need to use small mammals such as mice. Moreover, mice are macrosmatic and rely on scents to drive their behaviour, making the the ideal model to focus on how the environment shapes

plasticity, learning and behaviour in the olfactory and dopaminergic networks.

Which non-animal alternatives did you consider for use in this project?

I considered the following options:

- in silico models such as computer simulations
- invertebrates, such as worms (Caenorhabditis elegans) and flies (Drosophila melanogaster)
- human-derived stem cells
- brain organoids, which are three-dimensional clusters of stem cells resembling a small brain-like structure

These methods are used in other fields in biology and neuroscience which study processes happening in single cells, or simple behaviours in insects. Unfortunately they not suitable to answer the kind of questions that I am asking because I need to link changes at the cell levels with complex behaviours and pathologies.

I maximise the use of non-animal methods for many of my experiments. When doing so, I obtain brain tissue from mice that are painlessly killed.

Moreover, I am setting up a collaboration with computational neuroscience. The data gathered from the animal work included in this project will hopefully be instrumental to attempt to generate computer simulations of plastic olfactory network, to be used for future studies.

Why were they not suitable?

Our understanding of how the brain changes in response to changes in the environment is improving, but is not yet at the point where we can build realistic mathematical models to simulate and investigate different scenarios.

This means that we need a better characterisation of the mechanisms and effects of plasticity in real neuronal circuits, which is precisely the goal of the proposed research. Human-derived stem cells or brain organoids are not currently used for integrated investigations of multiple forms of plasticity in realistic neural networks, and human-derived cultures of the brains cells that encode for smells are also not currently available. In addition, of course, such cultures would not be able to address the multi-level analysis (i.e., from cellular changes to behavioural changes) which is central to this project's success.

In short, there are no viable alternatives to animal use if the project is to meet its stated 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?

Animal numbers were estimated based on my extensive experience with these experiments, by using previously-collected data, and by consulting with colleagues specifically trained in statistics.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I used online reference tools (NC3Rs Experimental Design Assistant), and made sure that my plan adhered to all best practices in experimental design. These include:

- using control littermates to minimize breeding mice that are not then used for experiments;
- including animals of same age and sex in all groups to minimize variability;
- randomised group assignation;
- blinding: the scientists will not know information which may influence or bias the results until all analysis is completed;
- appropriate statistical analysis (e.g., multilevel mixed model analysis with mouse as the subject variable).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

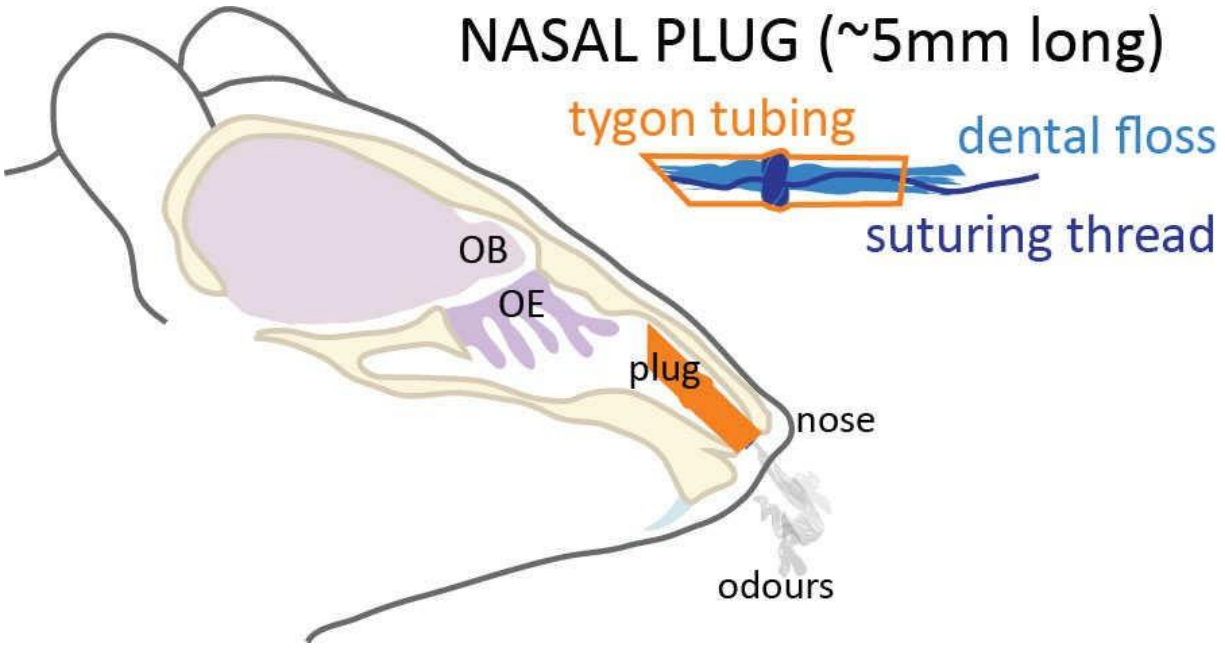
- Refinement of surgical techniques and of behavioural testing, as well as high quality animal welfare, will minimise suffering and distress. This in turn will reduce inter-animal variability and thus decrease the number of animals needed to achieve a meaningful result.
- Longitudinal recording of the same brain cells achieved via chronic imaging, which will reduce the number of used animals and increase statistical power.
- For new experimental approaches, small pilot studies to determine appropriate animal numbers.
- Efficient breeding system to minimize generation of surplus animals.
- Tissue-sharing among laboratory members, and, when feasible, with other laboratories.
- Generation of computer models based on the data that we collect.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

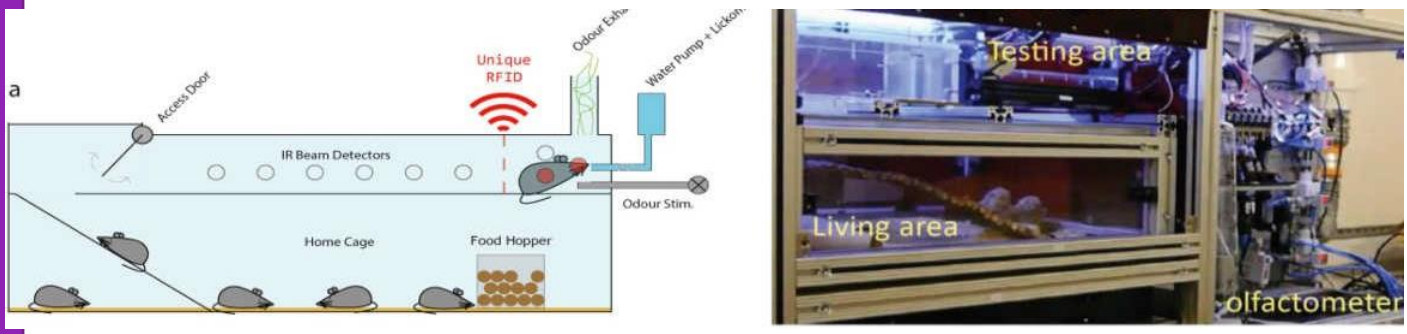
Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Sensory deprivation: this will be achieved by my choice of occlusion of one nostril via a removable plastic plug, rather than the painful and irreversible surgical nose closure typically used in this type of research. This technique is now routinely employed in the various laboratories. In addition, suffering will be minimised by carrying out the procedure under appropriate anaesthesia, preparing custom- made plugs of appropriate size which' as illustrated in the figure below, blocks the airflow without damaging the olfactory epithelium (OE) or olfactory bulb (OB) in the brain. Lubricant will be used to facilitate plug insertion.



Surgery: all procedures will be carried out aseptically in the designated surgical suites of the animal facilities. Appropriate anaesthesia and pain medication will be provided before and after surgery at doses and frequencies agreed in advance with the veterinarian. I have over ten years' experience in performing recovery surgery in mice, and I have had a very low incidence of complications. I will have in place clear protocols detailing how often the animal will have to be checked, how to assess pain and distress, and how to proceed if surgical complications occur (immediate humanely kill for serious complications, swift consultation with the veterinarian and intervention for minor complications).

Behavioural testing: traditionally behaviour has been tested in rodents by means of a go-no go task: water-deprived mice are presented with a series of smells/images/sounds, and only rewarded with a drop of water for some of them (targets). Mice learn reasonably quickly to lick the water spout exclusively when target stimuli are presented, and such lick measures are used to quantify performance, learning rates and memory consolidation. While extremely effective and well characterized, this methodology has important caveats – first and foremost, water deprivation. In order to overcome these limitations and to provide a more ethical way to test smelling behaviour in mice, a fully-automated testing apparatus has been recently developed.



Briefly, colonies of up to 20 mice of the same sex share a large two-story cage. On the lower floor they have access to nesting material, environmental enrichment and unrestricted food. When they want to drink they need to go to the first floor and access a separate compartment, where only one mouse at the time can enter after having its identification under-the-skin microchip read at the door flap (similar to house cats with their cat doors). Both the water port and the odour delivery spout are located in this separate compartment, and the mouse will have to perform an olfactory go/no-go task in order to receive water. Testing is therefore self-initiated, and mice will do as many sessions a day as they want. Mice are neither water restricted nor single housed: they do not drop in weight and do not appear stressed, and thus the performance variability across days and individual is reduced. Moreover, the live monitoring of how individual animals engage with the task guarantees that water intake will be closely monitored. If on any given day a mouse fails to engage with the task or makes lots of mistakes and thus does not drink enough, the experimenter will be promptly alerted by the software and water will be manually given to the animal to avoid dehydration. Animals do not live in the testing apparatus all their lives, but they are placed there with their littermates for the duration of testing, and then returned to their normal home cage.

Why can’t you use animals that are less sentient?

The choice of mouse as the animal model ensures that I am using the most appropriate and simplest species possible for the proposed research. How the brain processes smells, and how brain cells change their shape and function in response to inputs from the environment are all best understood in rodents, providing a wealth of background information that will prove essential for the project's success. In addition, many of the experiments outlined above would not be possible without the availability of key mouse transgenic lines.

Throughout my work I will address the 3Rs requirement for refinement by minimising animal suffering. Moreover, the majority of the work will be performed on tissue acquired from mice that had been killed under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be group housed and provided with appropriate enrichment such as nesting material and toy wheels, and I will adopt tunnel handling. To minimize the single housing of stud males, they will be kept in low numbers and rotated among partner females. Each female will not carry more than 6 litters, and no mouse will be kept for more than one year. In line with the HO GAA Framework, genetic identification of littermates will be carried out by collecting small ear samples for DNA analysis (second/third week of age); these small pieces of tissue are the by-product of identifying the mice and therefore by combining

identification and genotyping we will minimise procedure done to the animals.

For surgical interventions, we will employ pain relief medication, surgical care and observation sheets, use of grimace scale or other body scoring tools. Instead of the normal hard food pellet hanging from the cage ceiling, softened food pellets will be left on the gage floor after surgery, to facilitate eating during recovery.

Before behavioural testing, mice will be acclimatised to the new environment, and handled for a few days by the person performing the experiment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will routinely discuss care and husbandry with my Named Persons, check the 3Rs Search Tool, and keep updated on the PREPARE guidelines, ARRIVE version 2 guidelines and the 2017 LASA guidelines for surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly visit the NC3Rs website, the Norecopa website and meet with the named persons in my facility to ensure best practice. Moreover, I will regularly interact with colleagues and collaborators, in the UK and internationally, working on similar topics, to exchange notes and keep up to date with the latest developments.

109. Regulation of Th2 immune responses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - (iii) Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

allergy, helminth, infection, immunology

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 elucidate how Th2 responses are regulated during chronic infections and allergic disease. To use the knowledge gained to develop new approaches to treat infections and allergies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Humans can be infected by a wide range of different pathogens, including viruses, bacteria, fungi, and parasites. As these different pathogen types infect the body in different ways, the immune system has evolved different immune killing mechanisms to deal with the different pathogen types. So, humans have three types of immune response, referred

to as Th1, Th2, and Th17, that are specialized in dealing with different types of pathogens. Mounting the correct type of immune response is essential for dealing with infection, whilst mounting an incorrect immune response can result in disease such as allergies or autoimmunity.

This project focusses on understanding Th2 immune responses, which are essential for protective immunity to parasitic worms (helminths), but when they go wrong are also responsible for causing diseases such as allergies or asthma. Recently, it's been shown that Th2 responses are more complex than originally thought, and comprise a wide range of different sub-types rather than taking on a single phenotype. There is also evidence that mixed Th2 responses can develop that include elements of Th1 and Th17 responses. Research suggests that different subtypes of Th2 responses, and mixed Th2/Th1 and Th2/Th17 responses, result in different infection and disease outcomes. On top of this, infections and diseases associated with Th2 responses are usually chronic, lasting months to decades. There is evidence that Th2 responses can switch between different subtypes, or mixed phenotypes, over time which can then alter the severity of disease or susceptibility to infection. However, our knowledge in this area is limited, and we don't fully understand how these different types of Th2 response impact the outcome of infection and allergies, or what causes the Th2 response to switch towards different phenotypes.

This project aims to understand how and why Th2 responses switch between different subtypes, and develop mixed responses, during parasitic helminth infections and allergies, and to define how these switches influence susceptibility to helminth infection and progression of allergic diseases. This knowledge will help us to predict disease and infection outcomes. It will also identify new therapeutic approaches that may be used to manipulate Th2 responses to treat helminth infections and/or allergic diseases.

What outputs do you think you will see at the end of this project?

The primary output of this project will be new information that will improve our fundamental understanding of Th2 immune responses. The project will identify and perform some initial testing of pathways that have the potential to be therapeutically targeted to manipulate Th2 responses during allergic inflammation and helminth infection. This knowledge will have particular relevance to the treatment of helminth infections and allergies, but will also give fundamental knowledge on how immune responses are regulated that will have broad relevance to the field of immunology. This new knowledge will be outputted in the form of seminars and conference presentations, as well as through open access publications in peer-reviewed journals. I also perform public engagement activities, primarily school talks, and will discuss ongoing research from this project.

Who or what will benefit from these outputs, and how?

In the project's timeframe, knowledge will be the primary output, so the main beneficiaries will be the academic community. Primarily those working in the fields of helminth immunology and allergy, but the fundamental information garnered will also have broader relevance to all those performing immunology research. Our research does feed into our undergraduate and MSc teaching, and through publication engagement feeds into school education (e.g. advance highers), so does also have some educational impacts.

Identifying immunological pathways that could be used to therapeutically manipulate Th2 immunity could be of interest to the pharmaceutical companies for further development. This is likely to be towards the end of the project (years 4/5 of the project), and the years

immediately after the project (approx. 5 – 10 years post project).

It is possible that the information from this project could in the future lead to the development of therapies that result in human health benefits. However, the timeline for any such benefits would be in the decades (10 – 20 years).

How will you look to maximise the outputs of this work?

As the major output of this proposal will be new knowledge, dissemination of our research results to the academic community will be key goal for stimulating and developing new research. Each year we will attend a UK and a non-UK meeting, with the goal of publicising and research and developing new collaborations. We will select a mixture of specialised congresses on Th2 immunity to reach researchers studying allergies, fibrosis, wound healing, and helminth infections, and those with a broader remit (e.g. meetings on immune regulation) to ensure that our research is disseminated to a wide audience, including researchers studying therapies and immune regulation during cancer, autoimmunity and chronic Th1 driving infections. The results of this project will be disseminated to the academic community through publication of primary articles and reviews in peer review journals, oral and poster presentations at national and international congresses, and by giving seminars at UK and international universities. Large data sets (e.g. sequencing), and raw data, will be made freely available upon publication.

In terms of developing new therapies and maximising any commercial outputs, prior to publication, we will determine whether we should seek IP protection to ensure no disclosure of potential patentable outputs occurs. Dissemination of the results to, and establishing collaborations within, the wider academic community as detailed above will be an important step in identifying and approaching relevant commercial stakeholders (e.g. biotech and pharmaceutical companies). We will also directly approach relevant biotech and pharmaceutical companies. Rather than using third-party companies, an alternative approach to the development of new therapies will be to start our own spin-out company to develop potential therapeutics.

Through public engagement this project could influence public knowledge and engagement in science. We include the research in this proposal in local school talks, typically to 17-18-year-old biology students, given 1-3 times per year. To reach a wider audience, press releases will be prepared to coincide with any publications to disseminate the study to the general public.

Species and numbers of animals expected to be used

- Mice: 1320
- 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.

The proposed project is intended to extend our understanding of induction and regulation of immune responses during infection and allergic inflammation. The complex immune

responses invoked by infection and allergen challenge can only be studied in a meaningful way in the whole animal, as they rely on multiple specific cell interactions that are temporally and microenvironmentally restricted. It is not possible to recreate these conditions in vitro. We use mice because their immune system is similar to that of humans, and the wide range of immunological reagents and genetically altered strains (e.g. strains of mice with a specific gene deleted or inserted in order to assess the function of that gene) makes them the ideal species for precisely investigating and experimentally manipulating immune responses. Mice are permissive for helminth (parasitic worm) infections (which are obligate parasites and can't be maintained in vitro), and can be induced to undergo allergic inflammatory responses, so provide models in which to study in vivo immune responses during helminth infection and allergic inflammation.

Rats are the natural host for one of the helminth parasites we work with, Nippostrongylus brasiliensis. This is an obligate parasite requiring passage through its host to complete its life-cycle. We thus use adult rats to maintain the N. brasiliensis life-cycle.

Typically, what will be done to an animal used in your project?

Experiments to investigate Th2 responses will typically involve the induction of a Th2 immune response in wild type or genetically altered mice, either through infection with parasitic helminths (Litomosoides sigmodontis, Heligmosomoides polygyrus, Nippostrongylus brasiliensis), through the induction of allergic airway inflammation, and/or immunisation.

In order to functionally investigate the roles of specific immune cells or molecules, we will need to experimentally manipulate their function in some mice. This is done using three main approaches. (1) By the use of bone marrow chimaeras to restrict gene deficiencies to specific cell types. In this process a mouse's bone marrow is depleted via irradiation and then replaced with bone marrow from genetically altered mice. (2) Injection of cells and/or substances that can manipulate immune function. As examples, injection of antibodies can be used to block the function of a specific immune molecule or deplete a specific cell population. Alternatively, injection of a specific immune cell type can be used to artificially increase the numbers of that cell. (3) Inducible gene knockouts, in which a specific gene or cell is deleted when the mouse is exposed to a specific molecule. E.g. If mice are genetically altered so that some cells express the human diphtheria toxin receptor, those cells will be depleted when the mouse is injected with sub-clinical doses of diphtheria toxin.

In order to phenotype Th2 responses, and to assess the outcome of experimental manipulations, we need to be able to track immune cells and assess their function in vivo. As an example, to track Th2 cells we use a genetically altered fate reporter mouse in which Th2 cells become permanently fluorescently labelled when a mouse is injected with tamoxifen. Alternatively, to measure the ability of cells to divide in vivo, we inject mice with BrdU or other dyes that label dividing cells. To assess immune function, small volumes of blood may be sampled from peripheral veins (typically the tail) to measure antibody levels and phenotype immune cell populations. Blood sampling is also used to quantify parasite infection levels in the blood.

The project aims to study chronic Th2 responses and Th2 memory response. To study chronic Th2 responses, continuous helminth infections and allergic airway inflammation models need to be maintained for up to 90 days. To study memory responses, the initial infection or inflammation needs to be resolved to allow memory cells to mature. In this

case, the infection or inflammation will be allowed to run for 2-4 weeks and the animal rested for at least 3 to 4 weeks. After resting, the memory responses may be immediately phenotyped with no further procedures being performed on the animal, or the animal will be rechallenged with helminth infection or allergic airway inflammation. Memory experiments may therefore last for 4 to 6 months.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection of substances, cells, and parasites, as well as blood sampling, causes transient pain and discomfort at the injection site.

Helminth infections with Litomosoides sigmodontis and Heligmosomoides polygyrus are typically asymptomatic and do not result in adverse effects. Infection with high doses of Nippostrongylus brasiliensis can result in transient weight loss (approx. 7 days). However, we typically use low dose infection that do not result in adverse effects, and rarely use high dose infections. Allergic inflammation and immunisation are not expected to result in adverse effects.

Irradiation and reconstitution to produce bone marrow chimaera's can result in transient weight loss lasting 3 – 10 days. Irradiation also increases the risk of infection. The infection risk is mitigated by keeping mice in sterilised 'individually ventilated cages' that protect them from microbes in the environment, and by keeping animals on water containing antibiotics. Weight loss is mitigated by supplementing the diet with easy-to-eat food.

Exposure to tamoxifen to activate inducible genetic reporters can result in transient weight loss that can last for the duration of the treatment period in some mice. Treatment periods are limited to a maximum of two weeks and weight loss is mitigated by supplementing the diet with easy-to-eat food.

Expected severity categories and the 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% of animals will be used for the establishment and maintenance of genetically altered animal breeding programmes, and will fall into the sub-threshold severity limit.

Approximately 30% of mice will be used for experiments that do not involve irradiation, tamoxifen exposure, or high dose N. brasiliensis infection, and will fall into the mild severity limit,

Approximately 30% of mice will fall into the moderate severity category. This will largely be due to the activation of genetic tracking systems by exposure of mice to tamoxifen. Irradiation and high dose N. brasiliensis infections will be used infrequently.

100% of rats will be used for the maintenance of the N. brasiliensis lifecycle. These rats will receive sub-clinical doses of N. brasiliensis and will fall into the mild category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our studies rely on looking at the immune response to infection or other conditions in the context of the whole body. The immune response is complex, involving interactions between multiple cell types that are time and location dependent. The immune system is also not an isolated system and can be influenced by interaction with non-immune cells, tissues and organs. We cannot emulate these processes in vitro, and in our experience in vitro studies do not always accurately reflect in vivo. No alternatives for parasite migration through the body, or for studying the effect of manipulating immune responses on parasite survival exist. Similarly, it is not possible to recreate models of allergic airway inflammation in vitro. Thus, the vast majority of our experimental work requires the use of animals.

Which non-animal alternatives did you consider for use in this project?

Cell lines

Immune cells from human blood

Human tissue biopsies

Why were they not suitable?

Immune cells are programmed by the tissues in which they reside, and through the interactions they have with different cells they come into contact with. It is not possible to recreate these in vivo interactions using cell lines in vitro.

Whilst the immune responses seen in the blood reflect those occurring locally at an infection site or inflammation site, there are also often striking differences. Immune responses tend to be tissue dependent, and it is not possible to see these tissue dependent effects by sampling immune cells from the blood. Human tissue biopsies can provide a snap shot of the immune response in the tissue at a particular time, however, they do not allow the experimental manipulations that we need to perform to assess immune function. Also, tissue biopsies are only rarely available. In particular, there are very few patients infected with helminth parasites in the UK.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have estimated animal usage based upon my previous home office license, which used similar animal models and approaches. My larger grants have expired (MRC & BBSRC research grants), and I am currently applying for new MRC and BBSRC research funding to cover the work in this license. I have smaller interim funding that allows me to perform experimental work in order to complete manuscripts for publication, and to develop the project and feed into future grant applications. The animal numbers are based upon the smaller levels of interim funding, and will be increased when larger research grants are won.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible we perform in vitro experiments using in vivo generated cells, and this is factored into our experimental design. For example, immunization of mice can be used to generate large numbers of Th2 cells that can be used for in vitro experimental immunisations. This allows the immune response to be generated under natural in vivo conditions, but requires less mice than when performing in vivo experimental infections or allergic inflammation. By allowing us to perform the experimental manipulations in vitro, this also reduces the number of experimental manipulations that the mice undergo. We discuss our experimental designs and analyses with statisticians prior to performing experiments, and perform the relevant power calculations to ensure that the optimal number of animals are used. If the experimental approach is new then we will perform initial pilot studies to gain the data that will allow us to perform power calculations. We use a randomized design to reduce the number of non- experimental factors that could influence the results (e.g. cage effects).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When a strain is not being used for a short time period, minimal numbers are bred to maintain the line. Where feasible, we will freeze any strain that will not be used experimentally for greater than 1-year.

However, we have created unique GA mouse strains (e.g. IL-4CreERT2), and our colony is the only source in the world. As the success of cryopreservation and recovery cannot be 100% guaranteed we will maintain unique lines as live colonies.

We have now stopped maintaining the L. sigmodontis life cycle and will instead source parasites from our collaborators. This approach means that we no longer need to maintain a gerbil colony, perform gerbil infections, or use juvenile mice, which is a significant decrease in animal usage compared with my previous license.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models to induce Th2 responses: Helminth (parasitic worm) infections of mice are generally well- tolerated, and infections with Heligomosomoides polygyrus and Litomosoides sigmodontis are asymptomatic. Infections with high doses of Nippostrongylus brasiliensis can result in weight loss. However, we will use sub-clinical infection doses for the majority of experiments, and only use doses that cause adverse effects if absolutely necessary. Immunisation of mice to generate Th2 responses only causes transient discomfort at the injection site and is not expected to result in suffering. Induction of allergic airway inflammation (AAI) causes transient discomfort during injection of the allergen. Intra- nasal exposure to antigens will take place under general anaesthesia to reduce discomfort.

Assessing immune responses in vivo: Investigating how Th2 responses develop and change during immune challenge is a key readout of this project. Sampling of blood, which causes transient pain and/or discomfort without adverse effects, allows us to track immune responses and assess parasite burdens longitudinally without needing to cull the animal. To label and functionally assess immune cells in vivo we inject tracers such as BrdU, which is used to measure cell proliferation. High or prolonged BrdU exposure can interfere with bone marrow progenitor cells resulting in leucopoenia or anaemia, and cause damage to the intestinal epithelia resulting in diarrhoea. However, we use sub- clinical dosage routines that do not result in adverse effects. Injection of tracers such as fluorescent dyes does not result in adverse effects.

Inducible genetic models: We take advantage of inducible in vivo reporter systems to track immune cell functions in vivo, and inducible knock-outs to delete specific genes at a particular time point or in a specific cell type. Administration of tamoxifen is used to activate CreERT2 systems, and of diphtheria toxin to delete cells that have been genetically modified to express the human diphtheria toxin receptor. Prolonged or high dose exposure to tamoxifen interferes with estrogen and progesterone signaling pathways and can result in suppression of bone marrow lymphopoiesis, and high dose diphtheria toxin can kill murine cells as well as target cells. For diphtheria toxin we will use sub-clinical administration regimes that do not results in adverse effects. For tamoxifen we will use an administration regime that does not result in the more severe adverse effects listed above. However, the dosage required to activate the genetic systems does result in transient weight loss.

Immune modulation: To functionally investigate immune responses we need to experimentally perturb immune system functioning. E.g. Block and delete a particular immune molecule or cell, or expose an animal to that molecule or cell. There are three main approaches we use to manipulate immune responses. (1) Irradiation and reconstitution provides a useful tool allowing us to restrict gene deficiencies to particular cell types if cell specific GA mice are not available. Irradiated and reconstituted mice are more susceptible to opportunistic infections, and show a transient weight loss. To prevent infection mice are kept in IVC cages, and typically recover from weight loss within 7 – 10 days. (2) Administration of soluble substances can be used to experimentally block or stimulate immune pathways. These treatments do not usually result in adverse effects. However, interfering with host immune responses has the theoretical possibility of causing immune-mediated diseases, for example by neutralising immune down-regulatory networks. This is rare and has not occurred in our experience, but is a consideration when

using new interventions for the first time. (3) Transfer of cells to a mouse can be used to determine the function of a particular cell type. This does not typically result in adverse effects, although intra-venous injection can be associated with sudden unexpected death within seconds if cells injected intravenously aggregate. This has not occurred in our experience, and is typically less than 1%.

Why can't you use animals that are less sentient?

Although different types of organisms have similarities in their immune system, less sentient animals tend to have a more primitive immune system. Many aspects of the mammalian immune system are specialised to mammals, and so can only be studied using mammals.

An individual's immune system develops over time, and is different in post-natal and juvenile stages compared to adult stages. Thus, adult animals need to be used in order to study mature immune responses.

Chronic models of helminth infection and allergic inflammation last from weeks to months and so are not possible to perform under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In line with veterinary advice we will adopt the latest techniques in animal handling (E.g. cupping) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied.

Anaesthesia and analgesia will be provided where suitable (E.g. for humane restraint during intra-nasal application of allergens). To reduce infection risk, the best aseptic technique will be used during surgery (eg sterilization of instruments between animals, full surgical drapes), and immunocompromised mice will be housed in IVC cages.

In situations where weight loss is observed, animals will be given easy-to-eat food within the cage.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our institute employs a dedicated team of veterinarians that are continually seeking to improve animal welfare and refine animal use. We consult closely with this team and use the extensive resources provided on their website to ensure we are following current best practices. These resources include comprehensive guidelines and standard operating procedures for most common rodent procedures. We also consult the resources provided on the nc3rs.org.uk website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We work closed with a team of dedicated veterinarians that are continually seeking to improve animal welfare and refine animal use. We discuss with this team our experimental approach and the best approaches to animal experimental work. The team also provides a range of guidelines and standard operating procedures for common rodent procedures, as

well as comprehensive training in animal procedures. We attend organised 3Rs seminars and workshops, which highlight best practice and recent improvements.

110. Neural Circuits for Learning and Decision Making

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, Neuron, Decision making, Learning, Neuromodulators

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?

We aim to identify neuronal circuits that underlie learning and enable efficient decision making.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite decades of research on psychological and biological foundations of learning and decision making, the neuronal signals that guide decisions and shape learning have remained unknown. It is critical to understand how individual neurons and neural population process information important for decision making and use those to instruct learning. Understanding neural bases of decision making is a critical step in building mechanistic insights into why learning and decision making is impaired in psychiatric disorders.

What outputs do you think you will see at the end of this project?

Our outputs are scientific publications (to publicly disseminate our findings) and extensive datasets that we publicly release which can be used for further investigations.

In the shorter term this project:

- Deepen our quantitative understanding of neural circuits underlying learning and decision making. Concrete examples include understanding how different branches of dopamine circuit shape the process of learning and decision making and understanding how neural population in the frontal cortex encode specific aspects of decision process such as valuation and action selection.

- Contribute to the 3Rs because our large-scale electrophysiological tools as well as high-yield behavioural tools we have developed allows for recordings from a large population of neurons during precise tasks. As a consequence, fewer animals are required to generate the same amount of data compared to conventional methods. Moreover, the experimental data we generate will help to constrain models of neural circuits which will help to optimise future experiments, and reduce the number of animals necessary to obtain significant results. Our behavioural method is now being used in other laboratories in the UK and worldwide. We will continue to optimise this approach and develop it further to devise yet more efficient experiments generating more data per animal.

In the longer term this project:

reveals the relation between signals in specific neural circuit and specific aspects of decision making in behavioural tasks that we can run in mice as well as human subjects. Our results could thus shed light on novel therapeutic approaches that can eliminate specific behavioural symptoms.

Who or what will benefit from these outputs, and how?

Theoretical neuroscientists, psychologists and neurobiologists will benefit from insights into neural coding. Our work combines behavioural and neurobiological methods and therefore researchers acrosspsychology as well as neuroscience would benefit from our work. Moreover, by studying neural bases of learning, researchers across artificial intelligence fields would benefit from this work.

How will you look to maximise the outputs of this work?

We release our findings and datasets publicly using both traditional journal publications as well as using pre-print servers.

We will extensively collaborate with other researchers, in particular with those in computer science and theoretical neuroscience. We have a long track record in such collaborations. For instance, we have previously collaborated with computer scientists on our dopamine experiments, and based on the most our recent results, we have started an extensive collaboration with two theoretical research groups.

Species and numbers of animals expected to be used

- Mice: 6600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

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 intend to use mice because mice can be trained to perform relatively complex decision making tasks and are particularly suitable for high-count electrophysiology, imaging and optogenetic studies and the availability of transgenic animals expressing genetically-encoded indicators or actuators in specific neurons has enabled long-term recording and manipulation of their function. Fish or fruit fly would not be suitable due to the complexity of our behavioural tasks, and NHP would not be suitable due to limitations in neural circuit tools.

Adult mice (6-20 weeks) will be used because they can be trained to perform relatively elaborate decision making tasks, and perform hundreds of behavioural trials per day, which is necessary for rigorous analysis of neural data.

Typically, what will be done to an animal used in your project?

Breeding and genotyping

The mouse undergoes a recovery cranial surgery under general anaesthesia and in aseptic preparation (typically lasting 2 h).

The mouse is placed on water control and is habituated to the experimental apparatus by the experimenter for 3 days.

The mouse is head-fixed and is introduced to the behavioural task in which it collects liquid reward.

During the behavioural task, neural activity is recorded or manipulated using optical or electrophysiological methods for up to 16 weeks (typically 10 weeks).

The mouse undergoes terminal anaesthesia and is perfused for histological examination.

What are the expected impacts and/or adverse effects for the animals during your project?

Gene induction: weight loss (2 days)

Anaesthesia: slow recovery from anaesthesia (2-3 h, less than 5% of animals).

Recovery surgery: Local inflammation/infection (less than 5% of animals), post-operative pain/discomfort (less than 5% of animals), transient bleeding (1 min, less than 10% of animals).

Water control: weight loss (2-3 days at the onset of the water control)

Chronic implants: Infection around the implant (less than 2% of animals, 1 day), detachment of implant (less than 2% of animals, 2-3 minutes).

Chronic recording: Potential discomfort (1 minute) of connecting/disconnecting the implant to/from the recording equipment.

Chronic imaging: Breaking of the optic fiber (less than 2% of animals, 2-3 minutes), infection around the fiber (less than 2% of animals, 1 day).

Expected severity categories and the 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% of 5000)

Mice: mild (50% of 5000)

Mice: moderate (100% of 1600)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our project investigates the neural basis of Learning and decision making. At present, this can only be studied by using the brains of animals or humans, because our understanding of brain function is too rudimentary to generate realistic mathematical models for testing hypotheses. Functional brain imaging measures in humans lack the sensitivity to observe changes in the properties of individual brain cells in response to stimuli, decisions and rewards. In addition, an important aim of this project is to manipulate brain activity in a cell type-specific manner using optical stimulation methods, which can prove the causal roles of neural activity in driving decisions, a tool which is not available in humans.

Which non-animal alternatives did you consider for use in this project?

Human volunteers, Artificial computer models

Why were they not suitable?

Neural bases of decision making can be studied in human volunteers. However, functional brain imaging measures in humans have coarse spatial and temporal resolution and thus cannot observe changes in the properties of individual brain cells during decision making. Invasive recordings with high spatiotemporal resolution can be only carried out in animals. We also aim to use neural manipulation tools to study the causal roles of brain activity in driving decisions, which will not be possible in humans. Some aspects of decision making could be studied in artificial computer models. However, these models are currently too simplistic to reflect complexities of brain dynamics and signals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number is estimated after careful consideration of our breeding protocol, the required genetic profile of mice after breeding (i.e. many animals will not have required genetic profile), and the number of animals that each experiment will require. In doing so, I have consulted with several current PPL holders who use the same or similar mouse lines and keep a colony for experiments, as well as statisticians and many published papers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our behavioural tasks are optimised to yield large amount of behavioural data from each animal (~5000-20000 trials per animal) allowing us to perform powerful analyses on the data with very small number of mice.

Having multi-staged protocols, with several optional routes, will maximise the information gained from each animal, allowing us to link behavioural performance with the results of physiological studies in the same subject. Wherever possible, we will use a within-subject design, allowing each animal to act as its control. Advances in multi-site recording arrays have massively increased the data yield from each animal, resulting in fewer animals being used than with more traditional methods.

Our experimental preparations (thousands of behavioural trials and recordings from thousands of neurons) require complex statistics and does not rely on conventional power and animal number calculations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We routinely develop and use cutting-edge quantitative models and analysis. Our group members are highly skilled in this regard and have published novel statistical tools. We also seek advice from statisticians and collaborate with computational neuroscientists. Statistical tests are carried out as the data are collected in order to ensure that sufficient animals are used and that additional animals are not used unnecessarily once rigorous statistical significance has been reached.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 particularly suitable for high-count electrophysiology, imaging and optogenetic studies; the availability of transgenic animals expressing genetically-encoded indicators and actuators in particular neurons has enabled long-term recording and manipulation of their function.

Mice are suitable because they can be trained to perform relatively elaborate decision making tasks. Many such tasks are those that we were only able to perform in species of higher sentience: from 2014 till present we have spent significant efforts in brining those behavioural tasks into mice.

Water control: we can use water control to motivate mice for performing our decision tasks because it is a very precise and reliable way to deliver reward, resulting in hundreds of behavioural trials per day.
Importantly also, under water control, it takes a very short time to bring the animal's weight to normal, should in rare cases the weight loss has occurred. Water regulation is the most refined method for motivating mice under head restraint, while enabling automated stimulus presentation, behavioural read-out and reward delivery. We have extensive experience of using fluid rewards in rodents. Our data from more than 200 mice we tested over the last 5 years show that when mice perform well on behavioural testing, they will get sufficient reward to stay adequately hydrated but still motivated to perform the test.

Head fixation: The potential stress due to head fixation will be minimised by frequent handling and habituation to the apparatus. We will habituate the animal to the apparatus and head-fixation gradually over 3-4 days and will incrementally increase the head-fixation duration. Relatedly, recent publications (e.g. Juczewski et al 2020) have confirmed that stress level significantly drops after 5 days of head- fixation. Wherever possible, chronic recordings will be carried out in freely moving animals using tethered lightweight implants that are easily supported by the animal.

Electrophysiology/Calcium imaging/Optogenetics: Cellular imaging and optogenetic experiments generally require intracranial injection of a viral vector/tracer several weeks before imaging commences. We have recently established that the interval required for effective virus expression is shorter than widely believed to be, potentially reducing the duration of individual experiments.

Chronic measurements of neural activity (electrophysiology or imaging) requires head restraint, in which case individual recording sessions will last no longer than 3 h (typically approximately 1 h), depending on whether animals are performing a task or just passively receiving reward. The maximum total recording duration time under head restraint will be 100 hours. The impact on welfare is expected to be minimal as the animals will have been habituated to the situation. Electrophysiological experiments will be carried out with electrodes which are 0.07 mm thick causing effectively no damage to the brain tissue.

Of the various methods for interrogating defined neural circuits, we will prioritise the use of optogenetics as this offers the most scientifically refined approach for studying their function. Although a chronic implant is required, optogenetic methods allow for selective and reversible manipulation of activity, whereas other methods are typically less selective and/or cause permanent changes, which need to be controlled for with additional experiments. Electrophysiology/Calcium imaging/Optogenetics are not expected to cause

any lasting pain.

Why can’t you use animals that are less sentient?

The behavioural tasks that we study cannot be taught to animals less sentient than adult mice. We employ and develop behavioural tasks that require relatively elaborate processing of sensory information and reward value. Thus, these tasks cannot be performed by fish or fruit flies in a meaningful manner. In fact, till about 5 years ago, these behavioural tasks were exclusive to NHP and rats. We have worked hard to translate these behavioural tasks to mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will continuously refine our procedures based on our experimental observations, method papers regularly published in our fields as well as advances in our experimental technology. Examples of our recent important refinements are:

1. we were previously implanting optic fibers with the diameter of 0.4 mm into the brain for imaging and optogenetics. Advances in optics and virus efficiency now allow us to use fibers with the diameter as small as 0.2 mm while still having the same signal quality.
2. advances in electrophysiology is now allowing us to use electrodes that are 0.07 mm thick (a significant reduction in the electrode thickness and hence brain damage).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA guidelines, RSPCA, NC3Rs, ARRIVE guidelines, PREPARE

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our most effective ways to stay informed about 3Rs advances for us is through our local biological and animal facility which regularly and extensively communicate these advances.

The other source of information is NC3Rs portal that include extensive resources as well as various training events. Over the last years, I have attended in person 2 NC3Rs meetings, and we will continue attending these to stay informed about the most refined experimental procedures.

111. The neural basis of listening

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Hearing, Listening, Auditory Cortex, Multisensory integration, Hippocampus

Animal types	Life stages
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?

Listening, as distinct from hearing, is an active process shaped by our previous experience and current goals. The overall purpose of this research program is to better understand how the brain, and particularly the auditory cortex, function to facilitate active listening.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 basic research that will increase our understanding of how the brain processes sensory signals and how what we see can influence what we hear. Since the principle complaint of listeners with even mild hearing loss is the ability to discriminate speech sounds in the presence of background noise an understanding of how the healthy brain processes sound scenes may allow us to build better signal processing devices for hearing aids and cochlear implants. However, listening is an active process which combines hearing, attention and even vision to operate effectively.

This work may also provide benefits for technology: an understanding of how neural firing is decoded is fundamental in the design of any neural prosthesis or brain-computer interface. Understanding how the brain encodes sounds such as speech in the presence of noise will aid the development of speech recognition software and speech processing algorithms.

What outputs do you think you will see at the end of this project?

This research is fundamental science addressing the specific question of how the auditory brain supports listening and addresses more generally the question of how sensory signals are processed by the brain.

Outputs will be scientific publications which will report novel insights into how the brain processes sound. Additional outputs include publications and conference communications, code to perform analysis and computational modelling based on our data, and the data themselves, which will be freely available for other researchers to use as a replacement for further experimentation.

Who or what will benefit from these outputs, and how?

Benefits for Hearing Science:

- Major advances in understanding of the function of auditory cortex, a brain area which is poorly understood
- Advances in our understanding of how we attend to one source in a mixture
- Advances in how the auditory brain deals with background noise and competing sound sources
- Advances in understanding how auditory and visual signals are combined to facilitate listening Benefits for Neuroscience
- A knowledge of how neural activity patterns, on different spatial-temporal scales, might be ‘read- out’ for perception
- Advancing our understanding of how sensory information is combined across modalities
- Development of the ferret as an animal model for sensory neuroscience. The ferret has been a popular model for auditory scientists due to its excellent low frequency hearing and the ability to perform complex behavioural tasks. In the last 5-10 years there has been a growth in the use of the ferret model for studying other sensory systems and brain functions. By generating essential ‘normative’ data (for example on brain connectivity, or psychophysical thresholds) we are laying the foundations for the growing number of researchers seeking to use a non-rodent animal model. We have already helped a Dutch lab establish a ferret colony as an alternative to macaque monkeys.

Benefits for Health

- Since the principle complaint of listeners with even mild hearing loss is the ability to discriminate speech sounds in the presence of background noise an understanding of how the healthy brain achieves this task may allow us to build better signal processing devices for hearing aids and cochlear implants.
- Understanding when visual information can enhance hearing may be of therapeutic benefit to listeners with hearing impairments by allowing us to develop training strategies that use visual processing to optimize listening abilities.
- Many developmental disorders are accompanied by a failure to appropriately employ attentional mechanisms. Understanding the role of selective attention in shaping neuronal responses to facilitate active listening will provide novel and critical insights into brain function.

Benefits for Technology

- An understanding of how neural firing is decoded is fundamental in the design of any neural prosthesis or brain-computer interface. The computational analysis which will be performed on our neural data will explore potential decoding strategies and provide insights that may assist in such technologies.
- Understanding how the brain encodes sounds such as speech in the presence of noise will aid the development of speech recognition software and speech processing algorithms.
- A highly novel aspect of this research is work in animals freely engaging with sensory stimuli in their environment – understanding how the listening brain utilises the additional information available from making head movements and orienting towards sources of interest is key to developing auditory prosthesis and computer listening systems that will be successful in everyday listening conditions.
- Neurophysiological data and computational neuroscience have provided inspiration for artificial neural networks (ANNs) underlying search engines, robotics, advertising, social media etc. Advance these fields with approaches such as deep learning will benefit from our understanding of biologically occurring neural networks of the brain.

Benefits for animal welfare

- We have an active collaboration with animal welfare and behaviour scientists, in which we are seeking to understand behavioural markers of boredom in captive animals and their neural correlates, and which may lead to methods to alleviate boredom in captive animals.

How will you look to maximise the outputs of this work?

Results from these studies will be presented at international conferences and be published in peer- reviewed scientific journals. This work also has the potential to impact on human health and on everyday technology in the medium term. Where appropriate we will additionally issue press releases and work with media to publicise our findings.

A variety of open access resources (such as Wellcome Open Research) provide venues to host data that include the publication of null results. We will also utilise such venues to publish technical method.

We already collaborate with a number of other ferret groups to share expertise and best practice. Our scientific code is shared via github, and all data is made available to other researchers in open access repositories for further analysis and hypothesis testing. We also make available files for 3D printing models which we increasingly use to build elements of our behavioural, surgical and recording approaches.

Species and numbers of animals expected to be used

Ferrets: 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 choose to use ferrets as our animal model due to their excellent low frequency hearing and amenability to behavioural training. Human hearing is critically dependent on low frequency sounds: this includes speech, music and our ability to localise where a sound comes. These frequencies are too low for mice and rats to hear. Our work focuses on auditory cortex and like humans (and unlike rodents) spatial hearing is crucially dependent on auditory cortex in ferrets.

We use adult animals as we are interested in how the adult brain processes sound, and because we train them in behavioural tasks that can take many months to learn.

Typically, what will be done to an animal used in your project?

Animals will be trained in behavioural tasks that require that they make judgments about a sound. This might require that the approach the location of a sound source, or they may categorise sounds (for example was it an "u" or an "e"), or they may listen out for the occurrence a particular target sound (for example the word "instruments" in a sequence of other words). In order to motivate the animals we regulate their access to water in their home cage so that the majority of their drinking water is obtained during their twice daily testing sessions. Animals typically perform behavioural testing from monday to friday, with free access to water from friday night until sunday night, on a three weeks on, one week off schedule.

Once trained in a task animals typically undergo a single surgical procedure under general anaesthetic (receiving appropriate analgesia before and after the procedure) to implant devices that allow us to either record neural activity or perturb neural activity during task performance. These devices are very similar in principle to a cochlear implant or a deep brain stimulator that may be fitted to a patient with Parkinson's disease. Once recovered from the surgery we continue to test the animal in behavioural tasks but before each testing session we connect a cable to the device which allows us to record or perturb activity during behaviour. We usually work with each animal for between one and three years.

What are the expected impacts and/or adverse effects for the animals during your project?

Behavioural training requires regulating animals access to water. Animals that don't have free access to water can be reluctant to eat dried food and may therefore lose weight. To counter this we monitor their weight very carefully and provide them with additional wet food after testing.

Animals undergoing surgical procedures will typically take 3-5 days to recover and for some of this time they will be housed singly. After this time we are unable to detect changes in their behaviour relative to their pre-surgical norm while they are in their home cage, during their behavioural testing, in the way they interact with conspecifics or during their daily ‘playtime’. Sometimes the skin around the implant can become irritated and require cleaning. Other than this we find the behaviour of animals with implanted devices to be indistinguishable from other animals in our colony: they are equally playful, inquisitive and active.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Surgical procedures are both classified as moderate severity, therefore most (~90%) of animals will fall into this category. However this level of severity will only be experienced by each animal for a short period of time during which animals are closely monitored and supported with pain relief and additional food and rehydration fluids are made available.

Water regulation is also classified as a moderate procedure. Almost all animals will undergo water regulation so that water can be used as a positively conditioned stimulus.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project involves in vivo experiments on ferrets. Studying the neuronal process of listening can only satisfactorily occur in an intact animal where sound can be delivered through the ears and processed in the normal manner by the brainstem and where we can make simultaneous measurements of perception and neural firing.

In order to understand neural mechanisms we must record the activity of single neurons. This is necessarily invasive and is not possible in human participants.

To understand perception we must make investigations in brains that are actively processing sensory stimuli.

Which non-animal alternatives did you consider for use in this project?

Alternatives to in vivo animal work include:

- In vitro tissue
- culture In vitro
- slice work
- Computer
- modelling Studies in
- humans.

Why were they not suitable?

While complementary, used in isolation these alternatives have significant drawbacks :

- In vitro tissue culture; cultured cells lacks the complexity of an intact brain in terms of both the inputs (which come via the eyes and ears) and network dynamics
- In vitro slice work; brain slices maintain some of the complexity of intact brains but are devoid of their sensory inputs and broader networks, and it is not possible to use behaviour as an index of perception

- Computer modelling; while our work relies upon sophisticated computer modelling and simulations of neural populations, we can only obtain the data on which to perform such simulations from real neurons: the auditory brain is not well enough understood to be simulated accurately on a computer. Nevertheless, computational modelling is integral to all stages of our work and enables us to refine our hypothesis by allowing us to explore and test our data with almost unlimited models as to how the auditory brain might function to facilitate hearing.Modelling approaches also enable us to relate what we learn at the level of single cells and small populations of neurons to the macro-scale measurements made in human brain imaging experiments.
- Studies in humans; functional imaging methods can tell us which areas of the brain are engaged in a particular behaviour (and have provided essential direction for our scientific objectives), these methods lack the spatial and temporal resolution to provide information at the level of individual nerve cells, and therefore cannot tell us about the mechanism by which behaviourally-relevant signals are encoded in the brain or about the circuitry that underlies that processing.

Psychophysical tasks, where we assess the performance of human listeners in a task is a critical method for clarifying the sorts of behavioural tasks that we should perform in animals and for extending the behavioural data that we collect from our animal model. Moreover we are able to ask more sophisticated questions in human listeners (simply because we can instruct them in a task) which enables us to test hypothesis generated by the computational analysis of our in vivo data.

While functional imaging, assessments of human perception and computer modelling are not satisfactory replacements for animal work, the testing of human perceptual mechanisms, and computational analysis and modelling of neurophysiological and behavioural data are integral to every aspect of our experimental process. They allow us to generate testable hypothesis that run from human to animal work and back again thus allowing us to ask specific, directed experimental questions in animals using the minimal number of animals. This approach ensures that we use the minimum number of animals for the maximum scientific gain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 a total of animals used will not exceed 100. Typically an 'experiment', designed to address one or more objectives, requires between 5 and 10 animals and takes approximately 2-3 years. Over the course of 5 years, our lab (which typically has one new PhD student each year and 2-3 postdocs at anytime) will complete approximately 6 postdoctoral researcher projects, and 5 PhD student projects, giving a requirement for up to 100 animals.

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 animals that we use in any experiment we maximise the data obtained from each animal by using state of the art recording devices that enable us to sample 10s - 100s of neurons simultaneously. This not only increases the absolute amount of data but also enables experimental comparisons within animals (for example, comparing how two distinct auditory cortical fields represent sound) that might previously only have been possible across cohorts of animals. Because we are able to maintain our chronically implanted devices for long periods we are able to design our experiments such that each animal can contribute data to multiple researchers again minimising the absolute number of animals required.

We have determined adequate sample sizes with power calculations and used the NC3Rs EDA tool to plan 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 carefully design our experiments so that data can be collated and shared amongst multiple researchers each studying different aspects of the dataset. We work closely with computational neuroscientists to ensure that the complexity and richness of the combined electrophysiological- behavioural data that we collect is fully realised.

To optimise animal usage we always pilot new behavioural paradigms in a single animal in order to refine the training approach before rolling it out more widely and develop new methods in naive animals so that we stand the highest chance of success when using animals who have been trained in behavioural tasks.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Model Species

Their audible frequency range overlaps with humans. Unlike rats and mice, ferrets have good low frequency hearing, which is essential for studying human hearing as both speech and sound localization are crucially dependent on low frequency sounds. It is not possible to better match human hearing in an animal model without using a primate model.

They are highly suitable for behavioural studies . Ferrets can easily be trained to perform behavioural experiments by positive conditioning. We have extensive experience of this and have used it in studies of auditory and visual localisation, binaural unmasking, auditory temporal processing, pitch and timbre perception, speech discrimination, spatial processing and discrimination in noise. While it is possible to train rats and mice in sound discrimination tasks we cannot test phenomena such as pitch perception because of their audible frequency range. Moreover the type of tasks that a ferret can be trained to perform

is considerably more complex than those that a mouse can be trained on which are typically based on detecting a change in a repeating sequence of sounds rather than making a categorical judgment.

The auditory areas of the cerebral cortex in the ferret are also required for normal sound localisation, just as they are in primates, including humans. This is not the case, however, in rodents where removal of the auditory cortex does not result in a localization deficit.

Being larger, the ferret offers advantages over smaller animals for chronic recording. We are able to record from up to 64 electrode channels simultaneously – this does not simply increase our yield of single neurons (and thus reduce the number of animals we need to use) but also allows us to ask qualitatively different questions about the dynamics of neural populations. We are additionally able to record over periods of years rather than just months.

A wealth of data has already been collected by myself and another UK group, as well as our international collaborators, on the higher levels of the ferret auditory system. Our understanding of the anatomy and physiology of the multiple auditory fields is as good as, or better, than any of the other commonly used animal models in hearing research. An increasing number of research groups in the UK and USA now use the ferret as an animal model which enables us to share data and expertise in a way that minimizes the number of animals required and maximizes the benefit from any one experiment.

Techniques

We perform chronic recording of neural activity, and perform targeted alteration of neural activity in ferrets trained to perform listening tasks.

Most recording experiments have to use awake animals, so that we can monitor their behaviour at the same time. We have developed very light weight chronic implants for this purpose, which are well tolerated by the animals and allow us to record from freely moving animals rather than using head fixation. Our recording methods are designed to mimimise suffering and distress: all recordings are made while animals are unrestrained and freely moving connected only by a light weight cable or wireless transmitter. They are highly familiar with the testing environment and the personnel. Our animals experience water regulation but they are tested at standard times twice a day and rapidly learn that they will have access to water during these times and to wet food immediately after testing. Our careful monitoring ensures that when animals do not receive enough water during testing we are able to supplement their intake with water in addition to wet food.

Recording of neural activity

We constantly strive to optimise our methods to allow us to eliminate or minimise suffering of animals. Our ferrets live in social groups in specially designed cages where cages are linked via perspex tunnels which run along the walls of the room allowing animals to move freely around large areas.

Chronically implanted animals are group housed (with the exception of immediately post-surgery typically for a period of 2-3 days) and live in the same enriched housing as non-implanted animals. Animals have regular (typically daily, Monday - Friday) ‘play-time’ on the floor of the holding room providing further opportunity for exercise and additional social interaction (all animals are group housed). Due to the small number of animals that we

keep each animal receives individual attention and extremely close monitoring. Daily behavioural testing ensures that we know the personality of each of our animals and are therefore able to spot subtle changes in behaviour that might be early indicators of an underlying physiological or psychological problem. We are fortunate in having excellent technical, surgical and veterinary support within our animal facility.

We are actively collaborating with animal welfare scientists to optimise and refine enrichment for our animals. There is a strong network of neuroscientists using ferrets as an animal model who share methods and techniques to ensure that as a community we work together to minimise suffering and use the most refined approaches available.

Water regulation

Measuring auditory perception in an animal model poses a number of challenges, principally in training animals to perform tasks. Training may be achieved either by depriving the animal of food or water in their home cage, then rewarding them during training, or by providing aversive stimulation (electric shocks). While some research groups use aversive stimulation coupled with fluid regulation we have had great success with water regulation alone and only ever employ this method. We choose fluid regulation over food regulation for a number of reasons: (i) it is easily delivered in a large number of precisely timed small doses, which produces a larger number of trials, (ii) it is less visible and therefore less distracting to the animal, and (iii) chewing food will generate more noise, which could both interfere with our measurements of the animal's hearing abilities and introduce mechanical artefacts into our electrophysiological recordings. A large number of different deprivation/reward procedures for ferrets have been attempted by us and collaborators. These included partial deprivation (providing less water in the home cage, or withdrawing it only for a portion of the day), various food rewards, and sweet (sugar or saccharin) liquids without deprivation. Unfortunately, none worked as well as provision of water as reward and the regulation of free water provided during testing periods, accompanied by regular breaks from testing (typically 2 days off, 5 days on) total.

Sugary liquids also present a maintenance challenge since in our experiments many reward delivery units are used, in contrast to one for most primate behaviour. There is also little evidence that a fluid regulation regime is physiologically harder than food regulation (see Prescott et al. 2010).

This fluid regulation regime has been working successfully for 9 years in our lab, and was adapted from the methods used elsewhere for more than 20 years. These methods have been subject to constant refinement, including during the current license. Animals are tested two (or in some cases three) times daily until satiated. Water intake is monitoredand compared to an estimated daily amount based on the animal's body weight. Animals who fail to receive this much water during behavioural testing are supplemented with wet food (a mash made from ground pellets and water) and/or with a small volume of water. We qualitatively and quantitatively assess welfare by monitoring animals' demeanour (are they bright, alert, inquisitive, interested in treats, active when placed on the floor, sociable as is usual for that particular animal), water intake, weight, behaviour, performance (in terms of the number of trials performed daily and the level of performance) and assessing dehydration by skin-tenting. We use this information to individually adjust the reward

programme to maximise water intake for each animal within the remit of our scientific aims (for example, the volume of the reward on each trial, and the supplementation of additional water). Weight loss is restricted to a maximum of 12% of the starting weight (and rarely approaches this). Over the course of our previous licenses we have dropped this limit from 15% to 12%. Over the last few years we have collected and published a large dataset of weight measurements documenting seasonal variation (which is large, typically +/-20% in winter/summer) and their interaction with weight loss. This data allows us to be more refined in our use of weight change as an indicator of health.

Adapting to running experiments during 2020 with a minimal personnel presence in the lab has led us to develop a number of automated procedures to mitigate the decreased opportunities to communicate within the lab. For example, we have coded an automatic email generation feature to message a PIL holders if their animal receives less than expected water in a session.

Why can’t you use animals that are less sentient?

To study auditory perception we require that animals are engaged in a listening task to report their perception. This is not possible under anesthesia, and nor is it possible in a more immature life stage. To understand human hearing we need species that like us, have good low frequency hearing, ruling out many common animal models such as 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 implemented several refinements to our water regulation procedures:

We have established a normative dataset for seasonal female ferret weight changes. This is a dataset based on 39 animals weighed daily over 1-3 years. Seasonal variation was large, typically +/-20% in winter/summer, and interacted with weight loss such that weight loss on water regulation was greater in summer than winter. An important observation was that individual animals have highly stereotyped (across years) but variable (across animals) trajectories for weight loss and gain. This data allows us to be more refined in our use of weight change as an indicator of health and highlighted the need, where possible, to considering changes within an animal relative to their previously observed changes.

Adapting to running experiments during 2020 with a minimal personnel presence in the lab has led us to develop a number of automated procedures to mitigate the decreased opportunities to communicate within the lab. For example, we have coded an automatic email generation feature to message a PIL holders if their animal receives less than expected water in a session.

We are also refining the way that we review animals with implants. Currently animals are examined daily by lab members and animal technicians, and regularly by the NVS, and

formally reviewed for their suitability to continue for a further 6 month period (requiring a formal assessment, meeting with the NVS, NACWO and consent from the HOI) at 6 month intervals starting 12 months after an animal receives an implant. Instead of this ad hoc system of reviewing animals 6 monthly after implantation we will review all implanted animals simultaneously every 6 months and formalise this review procedure to include neurological tests. We will increase the ease of which we can observe changes in the animal's state by creating a photographic record of the animal's head immediately after surgery, after recovery and every 6 months thereafter.

We are refining our enrichment materials based on work (including a survey of nearly 1000 ferret owners and a series of behavioural assessments) performed in collaboration with an animal welfare PhD student.

We recently purchased a 3D resin printer. This has allowed us to self-manufacture implant wells and caps allowing the design to be much more bespoke to each animal. We hope the additional flexibility that this affords will allow us to produce minimally disruptive yet very robust implant caps.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow ARRIVE guidelines, and have used the NC3Rs EDA tool for experimental design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive information from the NC3Rs via my university and social media channels. Being embedded in a veterinary college has offered a number of opportunities that ensure we stay abreast of welfare developments. We actively engage with welfare scientists (I co-supervise a student working on boredom in laboratory animals), and when they occur, attend research conferences that link neuroscientists with welfare scientists. We work closely with the NACWO and NVS to implement welfare advances.

We also work closely with other ferret researchers through the 'ferret brain' network which connects researchers working throughout the world face to face every other year and exists as a virtual community. We share best practice including welfare advances and help each other to implement them successfully. For example I have helped several research groups (including some transitioning from primate work) to establish methods to implant electrodes for freely moving recordings, and a US group establish eye tracking methods in the ferret.

112. Brain-wide physiological mapping of neural circuits in mouse models of neurodegenerative 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

Dementia, Alzheimer's Disease, Neural Circuits, Two-photon microscopy, Electrophysiology

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 overriding objective of this project is to determine the effects of Alzheimer's disease (AD)- associated pathology on cells and circuits in the brain, and how this leads to cognitive deficits, behavioural abnormalities, and other disabling symptoms. We aim to follow the progressive decline of brain function before and during the development of AD-related neuropathology in the most natural and physiological circumstances. In so doing, we intend to provide valuable insights into the aetiology of Alzheimer's and other related brain diseases, and accelerate the development and application of effective therapeutic and curative approaches in these debilitating 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?

AD is a devastating neurodegenerative illness that affects millions of people and has become a leading cause of death world-wide, for which there are currently no effective treatments. For over a century, two pathological lesions –neurofibrillary tangles (i.e., aggregates of tau) and amyloid plaques (i.e., aggregates of amyloid-beta) – have been used to define AD pathologically, but the relationship of these pathologies to dementia remains unknown. Here we propose to test the hypothesis that plaques and tangles disrupt the function of cells and circuits in the brain causing progressive memory and cognitive deficits. This work will significantly contribute to our understanding of dementia through a multimodal focus on the earliest inducers of the disease, identification of common motifs across related brain pathologies, and the development and validation of potential therapeutic interventions which remain, to date, elusive.

What outputs do you think you will see at the end of this project?

We will gain new knowledge about the cellular and circuit mechanisms underlying progression in Alzheimer's Disease (AD) and related neuropsychiatric/neurodegenerative disorders. This is essential in order to develop effective therapeutic strategies to combat AD-associated dementia. The work described here represents a continuation and extension of work performed by us previously, which has resulted in many publications in high-profile international journals (e.g. in Nature and Science). The knowledge gained will be mainly communicated though pre-prints (e.g. BioRxiv), high-quality peer- reviewed, open access, publications and scientific talks, for which the PPL holder has a strong track record.

We will obtain large amounts of physiological and behavioural data that will be made available to the scientific community through publication and data sharing (data management plans in position as a requirement for Dementia Research Institute and external funding).

The results of these experiments, and the techniques we have developed, will provide new approaches for understanding dementia, and help inform therapeutic approaches and clinical trials.

Who or what will benefit from these outputs, and how?

The project goes to the heart of one of the main missions of the UK Dementia Research Institute – to understand the biological mechanisms of dementia. It holds out the possibility of understanding the cellular basis of the devastating impairment of memory and cognition in AD and a path towards translating that understanding to clinical practice. The new understanding and unique insights gained during this project will be extremely valuable to many other scientists and clinicians investigating neurodegenerative disease, and our studies will provide new avenues of research for future basic, translational and clinical studies in the long-term.

The project aims to identify novel therapeutic targets beyond amyloid-beta and tau, a major unmet medical need, and thereby drug-developing companies will benefit from this work. The data from our studies are potentially dynamic and sensitive markers of disease and thus provide a physiological readout of the effects of both disease and candidate therapies.

More generally, the UK will benefit by further raising its status as a world-leader in disease-related neuroscience and dementia, and help the Prime Minister's challenge on Dementia in succeeding to find a treatment for AD by 2025.

Another beneficiary of the project are also patients and the general public who will benefit through a better understanding of the fundamental mechanisms of dementia leading to the identification of novel therapeutic targets as well as an understanding of mechanisms underlying the failure of recent clinical trials, which might help change negative perceptions about AD drug development and increase public awareness about the urgency of research.

Charities and patient groups focused on curing Alzheimer's disease (e.g. the Alzheimer's Society and ARUK) and other dementias will benefit from greater insight into disease mechanisms.

Finally, staff will benefit from working on the project (e.g. postdoctoral fellows, and graduate students in my group). Involvement with this project could enhance, either directly or indirectly, their research and help them to develop a successful career in science. The range of approaches used, which include in vivo imaging and electrophysiology, offer unique opportunity for those involved to explore and expand their repertoire of skills and expertise, and their knowledge-base. It will also have a big impact on the work of our collaborators in the UK Dementia Research Institute.

How will you look to maximise the outputs of this work?

We will disseminate our work to national and international collaborators. We also have direct access to large consortia in Europe and USA that will enable rapid communication of relevant outputs at an international level. This will be achieved by presentation of results during visits or at conferences (virtual or in person, as the COVID situation allows) and through publication of results.

Furthermore, detailed descriptions of the research, accessible to both scientific and non-scientific audiences, will be added to laboratory webpages, and press releases will be arranged in arrangement with each University Media Team, if appropriate. We will also take part in public engagement events in order to communicate research to the wider community. Our university has long-standing experience of providing excellent public engagement training and award-winning public engagement and outreach events that will be exploited. State-of-the-art disease research such as ours can be framed in interesting and exciting ways and has clear societal impact, since so many people are aware of the ill-effects of AD. It can therefore be used as a tool with which to teach the community, and in particular children from disadvantaged backgrounds (e.g. through existing science communication events sponsored by our host institution), about the latest scientific research being conducted in their nearby educational institution, and inspire the next generation of scientists.

We will work with peer groups to organise, host and present at conferences and special journal issues that are likely to generate audiences consisting of experts in relevant fields as well as complementary areas.

We will participate in future events organised by professional and scientific bodies, such as workshops, mini-symposium and symposia meetings at international, national and local

meetings (virtually, if required due to COVID). This will allow the presentation of information to audiences including those from the pharmaceutical industry.

We will communicate via the University's media and public relations office that actively promotes the institution's research and undertakes media training for research staff, as well as through industry (pharmaceutical) publications.

We will communicate our findings (including negative results) via appropriate professional routes and networks that actively inform policy on healthcare and research such as Alzheimer’s Society or Alzheimer’s Research UK.

Should this project give rise to potential intellectual property this will be immediately discussed with the institution's IP Office to ensure full exploitation and translation of the findings.

Species and numbers of animals expected to be used

- Mice: 17500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use both wild-type mice as well as mouse models of human neurological disease that carry mutations or transgenes or express pathological proteins thereby causing brain pathology, abnormal brain function and/or cognitive and behavioural impairment. Since phenotypes manifest at different developmental stages and are often age-dependent and progressive (similar to human patients) we must investigate animals at different developmental stages and ideally longitudinally.

Mouse models of AD:

We use existing mouse models that develop Alzheimer-like plaques and tangles indistinguishable from those seen in the human disease. These mice thus allow us to observe the pathology, and surrounding cells and circuits directly to answer questions about how the disease progresses and whether various treatments allow recovery.

We will use well-established mouse models that express mutant forms of amyloid precursor protein (APP; with or without mutant presenilin 1 or 2 (PS1/2) which accelerate plaque formation) and deposit plaques in an age-dependent manner including the APP/PS1 or the APP-NL-G-F lines and similar models.

We will also use models that express tau and develop tangles in an age-dependent manner, such as the well-established rTg4510 mice that reversibly express tau and permit 'turning off' the transgene and assess whether deficits recover with the reduction in soluble tau. Similar tau lines include the rTg21221, the P301S, the THY-Tau22 or the htau models. We will also inject tau-AAVs, allowing us to limit the expression of tau to a subpopulation of cells. We can then test whether functional deficits are present only in those cells expressing tau or whether wider circuit disruptions occur when a small

population of cells express tau. This is an important question to assess the spreading of the effects of AD pathology and related tauopathies throughout the brain during disease progression.

Critically, Alzheimer patients have both plaques and tangles in their brain and it is thus important to not only assess the individual but also the combined and potentially synergistic effects of amyloid-beta and tau. In order to do this, we will cross-breed APP (with or without PS1) and tau models to produce mice that exhibit both plaques and tangles in their brains, such as the APP/PS1-rTg4510 line, which we have characterized in recent publications and know the time course of pathological development (see, for example, Busche et al. 2019, Nat Neurosci 22, 57-64).

Mice lacking tau or APP (and APP-like proteins) will also be used because reducing tau or APP levels has been found to protect against functional and behavioural phenotypes, and tau or APP-lowering is currently actively pursued as therapeutic strategy by a number of pharmaceutical companies.

We intend to cross-breed above mentioned models with existing transgenic lines in which genes of interest are expressed in specific cell types (e.g., Thy1-GCamP6), or mouse lines expressing Cre- recombinase (Cre) in specific cell types (e.g., PV-Cre). We will further inject a virally-packaged DNA fragment containing a gene of interest (e.g., the calcium indicator GCaMP) in an arrangement that means it will only be expressed in cells containing Cre.

Additional models of neurodege neration and neuropsychiatric disease:

In addition to the above, and in order to examine possible common motifs, biomarkers and treatment strategies across neurodegenerative and neuropsychiatric diseases, we will also seek to use additional models, such as those that recapitulate Huntington's Disease (e.g. zQ175), frontal temporal lobe dementia and amyotrophic lateral sclerosis (e.g. C9orf72), and autism and schizophrenia models. Due to the frequency of new advances in model development for such disorders, we will seek to employ those most aligned with our research question and which have been extensively characterised in order to ensure the translational relevance and reproducibility of our research.

Typically, what will be done to an animal used in your project?

Typically, after the breeding protocol(s), we will perform stereotactic surgery for targeted injections of viral vectors (e.g., Adeno Associated Vector, AAV) in the brain to express fluorescent reporters (e.g., the calcium indicator GCaMP or RCaMP) or optogenetic constructs (e.g., channelrhodopsin), and implant a cranial window with or without head-fixation apparatus. This will be followed by in vivo optical and/or electrical recording methods to functionally interrogate neural circuits at cellular level, both in anesthetised and awake animals, which is essential to quantify physiological function and dysfunction of circuits in the intact animal. We will also perform behavioural analyses in head-fixed and freely moving mice to assess the impact of circuit dysfunction on brain computation and thus the behavioural output of the animal. Furthermore, perfusion fixation and anatomical analysis, and/or acute brain slices, will be prepared for post-mortem in vitro analyses, which are crucial for obtaining the biophysical descriptors of individual cells' activity levels as the strength and dynamics of synaptic connections.

What are the expected impacts and/or adverse effects for the animals during your

project?

Most animals will undergo only mild procedures, with a minority experiencing a maximum expected severity of moderate. In these animals, adverse effects may include some post-operative pain, controlled by analgesia and some initial stress on head-fixation which will be limited by gradual habituation to the head restraint and provision of ample water, sugar-water or food rewards. Animals that are head-restrained in order to accurately image brain activity as per standardised protocols will be supported on a treadmill/wheel and are free to run or rest voluntarily, and are habituated gradually and systematically so they do not show signs of stress from the head restraint. Animals may lose small amounts of weight initially but will be supplied with supplementary gels/fluids to aid recovery and supportive food and treats throughout. In some cases, food or water restriction (without malnutrition or deprivation of essential nutrients) may be used to motivate animals to perform tasks, but will be strictly limited to the minimum necessary and closely monitored for adverse effects/ill-health. Any postoperative pain or complications that are not improved or resolved within a timeframe approved by the veterinary surgeon will be killed by a schedule 1 method. All animals will be killed by schedule 1 at the end of the experiment and their brains will be collected for further 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)?

Mouse models used under this licence will experience no greater than moderate severity levels. Approximately <40% of such animals are estimated to experience this maximum severity level, with the remainder expected to experience mild severities.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The main goal of the project is to examine the effects of neurological disease on cells and neural circuits in the functioning mammalian brain during sensory processing and/or a behavioural task. Currently no alternative exists to the use of animals for studying intact neural circuits with preserved neuromodulatory and sensory inputs. Mice are the lowest vertebrate species suitable for studying this and their biology and genetics are very well characterised. There is already a great deal of knowledge and an extensive publication history, that helps to optimize experimental design. We will use in vitro preparations where a suitable proportion of the circuitry is intact and where some elements of experiments can be better controlled. We also use advanced fixed tissue methods to examine circuitry anatomically at the microscopic level. Together, all of these approaches are valuable for informing the goals of the project and the data gained will help build more informed, larger

scale computer models to simulate neural circuit function in health and disease.

Which non-animal alternatives did you consider for use in this project?

Technology to simulate neural circuits is becoming increasingly powerful, however the parameters to constrain such simulations are still unknown, in particular in disease conditions where we still know very little. Human pluripotent stem cell cultures are still in their infancy and, while useful for some applications, represent only poor alternatives to the complex brain circuitry and behavioural repertoire present in animals. Direct human investigations are also currently not feasible, since there is no technology available that allows non invasive cellular-resolution recordings in the human brain.

Why were they not suitable?

This proposal aims to understand the effects of neurological disease on intact brain circuits and related cognitive and behavioural functions. This can only be studied in living experimental animals, because in vitro biological systems such as human pluripotent stem cell cultures lack the complexity of intact brain tissue with normal neuromodulatory and sensory inputs. These in vitro models cannot replicate the temporal and spatial distribution and composition of the pathological lesions, and do not mimic the adult organization of intact neural circuits. Moreover, cellular resolution is currently not possible in human brain, and mathematical models as well as computer simulations are not, as yet, suitable alternatives to live mice. As we learn more about cells and circuits under investigation we will be able to use mathematical modelling more extensively, but for such approaches to be useful they will need to be tightly constrained by biological measurements.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals required in this project are based on our previous experience of being project licence holders at other institutions, and populations estimated through comparison to existing and related PPLs held by other PIs at our establishment, with additional consideration of laboratory size and funding streams. Animal number estimates also take into consideration the requirement to breed different strains to produce double and triple transgenic lines. This is in order to model diverse pathological phenotypes (e.g. Alzheimer's Disease is a multiproteinopathy) and to understand cell specific contributions to pathoprogression (requiring cross-breeding of strains which contain markers of different brain cell types).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Number of animals used will be kept to a minimum required to allow statistically powerful, reproducible, valid and meaningful outcomes. For most quantitative experiments, design

will be based on PREPARE guidelines and sample sizes may be set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 20%. Otherwise we will use the minimum number of animals to provide an adequate description, generally on the basis of previous experience (our own and from the literature). We have extensive statistical expertise within our group and at the Institute to determine sample size based on power analysis, but we will readily consult experts in statistics if advice is needed, for example from our major collaborator (whose members have extensive experience with statistical analysis of data). We will work according to the ARRIVE guidelines to assure reproducibility of our research, and make full use of the NC3Rs EDA (as is already established and commonplace in our laboratory for any new research application) and other tools available on the website.

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 design breeding protocols to ensure that only the required number of animals are bred to minimise wastage. Mating occurs only to produce mice required for current experiments or to maintain a line for needs within the near future.

By recording from large numbers of cells simultaneously, we will use far fewer mice than would be required to record an equivalent size population individually. The use of high density electrodes (Neuropixels), two-photon imaging and mesoscopic imaging will allow recording of hundreds or more neurons simultaneously generating large numbers of data points per animal used. In addition to reducing the number of animals that must be used, these advances allow us to answer questions of neuronal coordination that would not be addressable only with single-cell recordings. Increasing the usable field of view size has also greatly enhanced the amount of data that can be collected from one animal. The recent introduction of dual colour imaging allows us to record changes in two variables of interest during an experiment (e.g. multiple cell types or calcium and glutamate signalling), reducing the number of animals needed and reducing the impact of cross animal variation. Data sharing across collaborative projects (e.g., within the UK Dementia Research Institute) will also reduce the number of animals needed.

Whenever possible we aim to perform repeated recordings in individual mice, not only reducing the total number of animals but also allowing important insights into the longitudinal trajectory of neurological disease. In addition, data from a given experiment may be used for multiple studies, including for computation modelling work which will feedback on research targets thus reducing the number of experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We aim to contribute to the understanding of human neurological disease; thus we have to

focus on a species that is both phylogenetically close to humans and genetically tractable. Rodents are probably the lowest species for which direct comparisons can be made with the structure and functioning of the human brain. Our primary experimental model system is the mouse, which is currently the species of choice in most areas of biomedical research, as it allows the use of powerful techniques such as targeted genetic manipulations to create models of disease. This has also enabled the selective expression of specific molecules, such as fluorescent proteins, in identified populations of neurons to facilitate target-directed recordings instead of random target selection. The levels of sophistication for targeting expression selectivity is constantly increasing, providing for further refinements in our approach. For example, recent advances will enable us to start selectively expressing fluorescent indicators in sub-regions of cells (e.g., only the soma of neurons), which removes the problem of 'contamination' by off-target signals (e.g., from neuropil), thus providing more interpretable data. We are working hard to implement 'online' (i.e. during the recording session) analysis which helps to shorten the animals' time on the set up and total number of sessions.

Although sensory responses can be tested under anaesthesia, it is well known that anaesthetic drugs affect activity in neural circuits and may provide misleading results. For this reason, many experiments, as well as those requiring a task performance, will be done in the awake animal. Core approaches used will be calcium imaging either in single cells in populations of neurons and whole-cell patch clamp recording, required to detect changes in the cell membrane potential and to detect synaptic current. To be successful, both of these methods require excellent stability and minimal movement of the brain. For this reason, the experiments need to be carried out on head-fixed animals. Head-fixation can be stressful for the animals, however we are taking several measures to minimize discomfort including the progressive acclimatisation to the behavioural rigs, so that the animals are not scared of spending time there and the use light-weight headposts, so that the animals can move normally when in their home cages. In addition, where possible to achieve our experimental aims, we will also be carrying out some of these experiments in freely moving animals using optical fibers coupled to miniature head-mounted cameras. We always use appropriate anaesthetic and analgesic regimes for pain relief during surgery, and much of our techniques in subsequent experiments are non-invasive, as they involve imaging through the skull or through a cranial window. Even when we do insert probes into the brain, these are fine probes, and they cause no pain as the brain has no somatosensation.

Typically, and whenever possible, we will group house mice and provide environmental enrichment, which further reduces stress levels significantly compared to isolated housing. With the help of the biological research facility, in particular the named animal care and welfare officers and veterinary surgeon, we constantly monitor best practices and new information available in the international literature and on the NC3RS and RSPCA websites.

Why can't you use animals that are less sentient?

Rodents are probably the lowest species of animals for which direct comparisons can be made with the structure and functioning of the human brain. Moreover, the sequence homology in genes of interest for dementia is minimal between invertebrates and humans and these invertebrate orthologues often lack regions of these genes that are important in dementia pathophysiology. The most notable example is the lack of amyloid beta in Drosophila and C. Elegans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our research depends on the animals being healthy, cooperative and engaged in the tasks that we have trained them to perform. We have thus scientific and ethical reasons to take any possible steps to avoid any suffering. We always use appropriate anaesthetic and analgesic regimes for pain relief during surgery, and much of our techniques in subsequent experiments are non-invasive, as they involve imaging through the skull or through a cranial window. Even when we do insert probes into the brain, these are fine probes, and they cause no pain as the brain has no somatosensation. Other measures that we take to minimise discomfort include the choice of light-weight headposts, so that the animals can move normally, careful controls on the minimum amount of water received every day, to prevent dehydration, progressive acclimatisation to the behavioural rigs, so that the animals are not scared of spending time there. In addition, we are extending our research to use novel lightweight head mounted microscopes that enable high-resolution imaging during normal behavior and free movement exploration. These techniques underscore our commitment to exploiting the latest in vivo technologies which improve the welfare of animals and improve scientific outcomes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The following published guidance will be followed to ensure experiments are conducted in the most refined way:

- UK government website
    - https://www.gov.uk/guidance/research-and-testing-using-animals
- Animals (Scientific Procedures) Act 1986
    - https://www.gov.uk/guidance/guidance-on-the-operation-of-the-animals-scientific-procedures-act-1986
    - https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_da ta/file/116860/quick_start_guide.pdf
- NC3Rs
    - https://www.nc3rs.org.uk/the-3rs
- LASA
    - https://www.lasa.co.uk/wp-content/uploads/2018/05/Position_3Rs.pdf
- RSPCA
    - https://science.rspca.org.uk/sciencegroup/researchanimals/implementing3rs

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

With the help of the biological research facility, in particular the named animal care and welfare officers and the veterinary surgeon, we constantly monitor best practices and new information available in the international literature and on the NC3Rs, RSPCA and LASA websites. We have attended, and will continue to attend, any relevant talks and seminars (particularly those for example hosted by our establishment's NC3Rs regional programme manager) in order to implement the latest best practices.

113. Investigation of genetic forms of neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

ALS, FTD, neurodegeneration, neuroscience, dementia

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the underlying mechanisms of neurodegeneration and develop therapeutic strategies for treating neurodegenerative diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Nearly all neurodegenerative diseases are currently incurable and present devastating outcomes for patients and caregivers. As the average age and size of the population continues to increase, so does the incidence rates of these diseases, making development of treatments for neurodegeneration an urgent public health priority. The purpose of this work is to investigate the underlying biological mechanisms of neurodegeneration that can be ultimately translated into therapeutic strategies for patients.

What outputs do you think you will see at the end of this project?

The expected benefits of this research will be a greater understanding of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) – two devastating neurodegenerative diseases for which no effective therapies exist.

Specifically, we will further understanding of FTD and ALS caused by the C9orf72 gene (termed C9FTD/ALS), which is the most common known cause of FTD and ALS. The new models we will generate should be appropriate for testing new therapeutics. Therapeutic strategies we are testing will help us understand mechanisms of neurodegeneration while also having the potential to translate directly to therapeutic strategies for patients. This will be extremely worthwhile for alleviating patient suffering and improving human health.

Additionally, new reagents and findings will be made publicly available to the research community through publication and dissemination to public databases and catalogs.

Who or what will benefit from these outputs, and how?

In the short term, the research community will benefit from this work, as it will lead to a better understanding of the underlying mechanisms of disease. This will generate new hypotheses for further study. Additionally, new mouse models we create will be made available to the research community which will further their own studies and unlock new findings in the future.

In the long term, the ultimate goal is to develop treatments for FTD/ALS which can be translated to the clinic and used to combat neurodegeneration in patients.

How will you look to maximise the outputs of this work?

This work will be published for the scientific community. As a rule we make all new mouse models available to the community after first publication, and therefore we will also be contributing resources for neurodegeneration research. We also make our models available before publication through collaborative agreements, to further their use and the knowledge they generate. This includes specifically designed and tailored mouse models that take a considerable investment of expertise and funding to produce, but which may be extremely valuable to the community for tackling specific questions in neurodegeneration. Importantly making our models available should reduce the number of new mouse models that would be generated independently.

Species and numbers of animals expected to be used

- Mice: 19325

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 as their genetics, physiology and neuroanatomy are very well

characterised and their breeding time and lifespan allow experiments to be performed on a manageable and affordable timescale. Their similarity to humans in terms of genetics and neuroanatomy, and their genetic tractability, which allows for the generation of genetic models of human diseases, has led to the generation of many successful models of neurodegenerative disease and the identification of potential therapeutic avenues. Furthermore, the phenotyping tests available for investigating neurodegeneration are well characterised. For these reasons, the mouse is our model of choice for gaining new insight into neurodegenerative disease mechanisms that are likely to be relevant to humans.

Neurodegenerative diseases are late-onset diseases. For this reason, we typically assess at adult timepoints.

Typically, what will be done to an animal used in your project?

To support our programme of research we will breed and maintain genetically modified mice (protocol 4) and wildtype mice for comparison. This will include reconstitution and freezing down embryos of relevant lines (protocols 1-3), including transgenic and knockout GA mice with relevance to neurodegeneration. We will utilise a range of approaches to introduce genes that cause dementia or amyotrophic lateral sclerosis, such as AAV-mediated gene delivery (e.g. intracerebroventricular (i.c.v.), intrathecal (i.t.), intracranial (i.c.), intravenous (i.v.)) or standard genetic breeding, in order to generate models of these diseases. As we are investigating age-related neurodegenerative diseases, mice harbouring these genes will be aged up to 2.5 years of age to investigate how neurodegeneration occurs. We will treat mice with potential therapeutics via a range of routes these include neonatal i.c.v.,
i.t., and i.c. AAV transduction (protocol 7), as well as intraperitoneal, oral gavage, i.v., and compounds made up in chow or water (protocol 6). Treatment will be performed at a variety of intervals at different stages of the disease depending on the nature of the agent. Following treatment mice will be phenotyped to assess the effects of the treatment, or killed for tissue sampling and analysis. As the genes we are investigating cause both dementia and amyotrophic lateral sclerosis we have included tests that will detect both cognitive and motor phenotypes. This is essential for us to determine the effects of the genes and drug treatments that we are investigating.

What are the expected impacts and/or adverse effects for the animals during your project?

- Female mice for superovulation will be of an appropriate size, in particular if they are to be mated. Over-vigorous males will be replaced.
- Animals undergoing surgical procedures carried out aseptically under general anaesthesia may rarely develop post-operative complications. Such animals will be killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery.
- Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Any animals that fail to do so or exhibit signs of pain, distress or of significant ill-health will be 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) will be killed by a Schedule 1 method.
- As we are investigating neurodegenerative diseases, some mice will develop early

symptoms of neurodegeneration such as impaired cognition or reduced motor function. These mice will be monitored regularly and killed at pre-defined humane endpoints.

- Neonatal animals will be injected with AAV via 1) intracerebroventricular (i.c.v.), 2) intracranial (i.c.), or 3) intrathecal (i.t.), routes, all of which are described in Marshall et al., Molecular Therapy, 2018. After treatment animals will be allowed to recover completely in a warm environment before the litter is returned its home cage. After recovery, animals will be maintained and weaned as normal. After the procedure all animals will be monitored closely for signs of infection or distress, in which case the NVS and/or NACWO will be consulted, and the animals may be killed by a schedule 1 method if no treatment is 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)?

Based on the predicted severity and numbers of mice for each protocol, we expect 55% of mice to be sub-threshold, 15% to be mild and 30% to be moderate.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We still know little about the pathways and critical mechanisms involved in neurodegeneration because of the specific difficulties in obtaining essential tissues: for example, the human post mortem samples we obtain are extremely limited in numbers and tissue types, and come from the final, endstage, of disease – and thus cannot tell us about the early processes that lead to the death of neurons. There is no alternative to studying the full consequences of neurodegeneration, and neurodegenerative gene mutations, in the whole animal.

Which non-animal alternatives did you consider for use in this project?

We work as much as possible by analysing human data and resources, including extensive use of post mortem samples and induced pluripotent stem cell (iPSC)-derived models. We also routinely use Drosophila models.
These alternatives allow us to replace some live mouse experiments, particularly in terms of refining experimental approaches and potential treatments before embarking on experiments using mice.

Why were they not suitable?

Currently primary, iPSCs, and other cell models are helpful, but cannot recreate the complexity of a whole body, or even an intact multicellular brain, including the effects of ageing. In addition, we now know that the death of many neurons depends on what is happening with the supporting non-neuronal cells that surround them in the brain, spinal cord, and periphery. Drosophila are a powerful model, which we have used to identify new genes that can be targeted to ameliorate neurodegeneration.
However, to be able to take these new findings forward towards therapy development in humans, it is essential that our Drosophila work is now complemented with a mammalian 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.

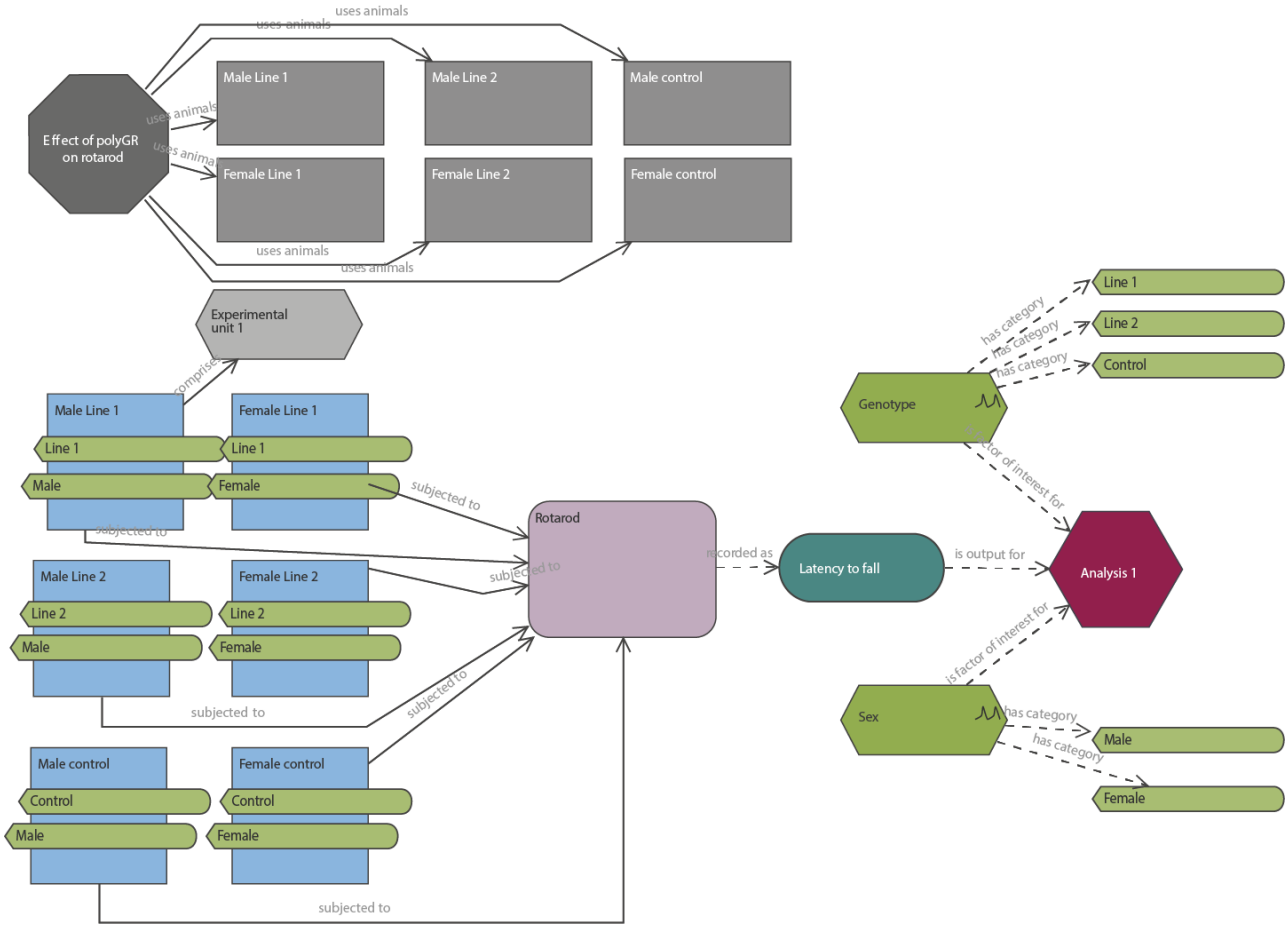
How have you estimated the numbers of animals you will use?

Every effort has been made to estimate reasonably the number of animals that will be required for each section of this project. In each case, maximum numbers are given and we may find within the course of the project that the same result can be attained by using fewer animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will try to reduce numbers of animals used by combining tests to be carried out on any group of animals, within Home Office guidelines, although we must remain cautious of the fact that exposure to one particular test may affect the performance in another. We will take expert guidance regarding possible combinations of tests.

We are now using the NC3Rs Experimental Design Assistant (EDA) to aid in experimental design and analysis. As an example, for rotarod analysis of our new lines, EDA recommended a two way ANOVA.



Based on the EDA recommendation, we used GPower to perform our power calculation. For an effect size of 10% deviation from the group mean, with a power of 0.85 and an alpha of 0.05, groups sizes of 28 are needed, note this is a combined group of both males and females (i.e. 14 females and 14 males per group). Sample sizes will be refined using EDA and power analysis once the standard deviation is obtained for each test with each specific mouse line. We also have access to advice as needed from local statistical experts.

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 embarking on a breeding project the absolute number of mice required for the phenotyping assay will be determined, in order to optimise our breeding strategy. The minimum number of breeding cages will be used to maintain the colony. We have considerable experience in maintaining lines using a minimum number of stock animals. Primarily this is achieved through assiduous monitoring of our colonies. Mouse lines will only be maintained whilst there is a justified use for their continued 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.

Choice of species

Mice will be used as their genetics, physiology and neuroanatomy are very well characterised and their breeding time and lifespan allow experiments to be performed on a manageable and affordable timescale. Their similarity to humans in terms of genetics and neuroanatomy, and their genetic tractability, which allows for the generation of genetic models of human diseases, has led to the generation of many successful models of neurodegenerative disease and the identification of potential therapeutic avenues. Furthermore, the phenotyping tests available for investigating neurodegeneration are well characterised. For these reasons, the mouse is our model of choice for gaining new insight into neurodegenerative disease mechanisms that are likely to be relevant to humans.

Phenotypic tests

Where possible non-invasive tests only causing temporary discomfort will be performed. Multiple testing on the same mice will be completed using the least invasive techniques and will only be continued if the mice show no adverse effect (e.g. excess stress, prolonged abnormal behaviour) to the previous test.

Minimising suffering

We do not wish to keep animals to the equivalent of end stage of disease that occurs in humans and so we will kill mice humanely before they reach this point. As such we do not have any severe protocols. We note that we are endeavouring to work, where possible, with mice that express mutant proteins at endogenous levels, unlike classical transgenic mice, and these ‘knock in’ and similar models tend to have milder phenotypes than standardly used models.

Why can’t you use animals that are less sentient?

Whenever possible we do use cell culture or Drosophila assays for our studies, but there are still some aspects of FTD/ALS disease research that are only possible in animal models. For instance, only mouse models will allow for the assessment of complex behaviours, such as anxiety and fear, for which there are deficits in a C9orf72 model (Chew et al, Mol Neurodegen 2019).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

By using anaesthesia and analgesics as necessary, and through careful monitoring, animal suffering will be reduced to an absolute minimum. We will also minimise harm by implementing high standards of care for each animal, by ensuring that the maximum severity for all protocols is either mild or moderate and by using well-defined humane end-points. Welfare assessment is essential in all projects involving animals and will be carried out, and recorded, as appropriate, throughout the lifetime of the mice. The following practices are well established at our facility:

- Neonatal assessment: when homozygosing a mutant for the first time, the neonate will be assessed from birth.
- Adult assessment: Using a standard vocabulary, health and welfare problems are documented and addressed.
- Phenotyping cohorts: Throughout phenotyping tests, mice will be monitored for adverse effects and fitness to continue in testing.

We are committed to using newer, more refined tests, such as home-cage monitoring systems, as they become available and feasible to implement.

General constraints

Please note, constraints on procedures involving anaesthesia, surgery, substance administration and withdrawal of fluids apply to all protocols.

Anaesthesia

Induction and maintenance of general or local anaesthesia, sedation or analgesia to mitigate the pain, suffering or distress associated with the performance of other regulated procedures is indicated using anaesthetic codes in protocols.

General anaesthesia

If authorised in this licence and unless otherwise specified, all animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health should be humanely killed unless a programme of enhanced monitoring and care is instituted until the animal fully recovers.

Surgery

If authorised in this licence and unless otherwise specified:

Surgical procedures should be carried out aseptically, to at least the published Home Office minimum;

In the uncommon event of post-operative complications, animals will be humanely killed unless, in the opinion of a veterinary surgeon, such complications can be remedied promptly and successfully using no more than minor interventions. Minimally inflamed wounds without obvious infection may be re-closed on one occasion within 48 hours of the initial surgery. In the event of recurrence, NVS advice will be followed;

Peri and post-operative analgesia will be provided; agents will be administered as agreed in advance with the NVS;

All animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health 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.

Administration of substances and withdrawal of fluids

If authorised in this licence and unless otherwise specified, administration of substances and withdrawal of body fluids will be undertaken using a combination of volumes, routes, and frequencies that of themselves will result in no more than transient discomfort and no lasting harm using published guidelines on minimal severity.

Appendix 1. Table of doses.

Dosing Table						
	Route and Volumes (ml/kg)					
	i/c¥	s/c	i/p*	i/m	Oral gavage	i/v bolus infusion
Mouse						
i) max no. of doses	2	24	28	6	20	14
ii) max daily volume	20µl	20	20	0.05ml	(See below)	5
No. of doses daily for ≤ 7 days		3x	2x	2x	2x	2x
No. of doses daily for ≥ 7 days		2x	1x	1x	1x	1x

NOTE: This dosing table does not apply to injections for animal welfare.

(s/c, subcutaneous; i/v, intravenous; i/p, intraperitoneal; i/m, intramuscular; i/c intracerebral)

*i/p refers to administration via injection.

These values are in accordance with ‘A Good Practice Guide to the Administration of substances and Removal of Blood, Including Routes and Volumes’; J. App. Tox., 21, 15-23, 2001.

¥ Volumes and frequency for i/c not available in either standards table quoted and therefore decided on the maximum volumes already in use by us and other laboratories. Maximum twice with minimum interval of 1 week.

Oral Gavage

Animals should be a minimum of 3 weeks of age before oral gavage. Volumes administered should not exceed 20 ml/kg.

Details of Injections

Needles of the smallest possible gauge are to be used.

subcutaneous injection into one or both flanks or under the loose skin around the neck.

For intravenous injections the tail vein will beused.

intraperitoneal injection into the abdominal cavity

intramuscular injection into anterior thigh.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to follow the NC3Rs' ARRIVE guidelines as best practice to ensure that our experiments are conducted in the most refined way. We will use the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery guidance https://www.ubs.admin.cam.ac.uk/files/lasa_aseptic_surg.pdf and the LASA guidelines on doses for each routes of administration

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have appointed a 3Rs Champion who leads our efforts to comply with all aspects of the 3Rs. This includes arranging regular 3Rs themed seminars from leaders in the field to stay up to date. The 3Rs Champion also collates information coming out of the NC3Rs and other relevant sources, and reports on 3Rs advances to our Animal Scientific Research Committee, which oversees our animal research. Where possible relevant advances will then be incorporated into practice.

114. Investigating how brain immune responses drive behavioural symptoms associated with mild and repeated traumatic brain injury

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Mild Traumatic Brain Injury, Concussion, Immune system, Inflammation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to understand how the immune system responds to a mild traumatic brain injury (mTBI) (also known as concussion), and repeated mTBI (rmTBI; two injuries) and how it affects post-injury symptoms.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Mild Traumatic Brain Injury (mTBI), often referred to as concussion, is the most common form of traumatic brain injury due to accidents, assaults, domestic violence, contact sports and war.

The immediate effects of mTBI are well recognised, such as a brief loss of consciousness, headache and even short-term memory loss. However, it is now understood that in the

long-term, mTBI, and particularly repeated mTBI (rmTBI), can cause mental health issues such as anxiety and depression, or even dementia and neurodegenerative disease in later life.

We are beginning to understand that mTBI is more dangerous than we thought, though we still don't know why.

Animal models suggest that the immune system can play a pivotal role in the both the damage and recovery of the brain after injury and during disease. However, much less is known of what specific role the immune system plays in mTBI. We hypothesise that an excessive immune response to mTBI and rmTBI is responsible for some of the long-term symptoms after head injury.

Here, we have an opportunity to understand the underlying biology of mTBI and rmTBI and pave the way for treatments in those suffering the long-term consequences.

What outputs do you think you will see at the end of this project?

Outputs from the project:

we will gain greater understanding of the nature of the immune response to mTBI and repeated mTBI (rmTBI) in a mouse model relevant to human injury.

we will understand if this immune response can be modified to improve behavioural symptoms after injury.

we will publish the results in open access, high-quality scientific journals and present the work at relevant conferences

we will share our results with collaborating scientists and ultimately make our data publically available

Who or what will benefit from these outputs, and how?

As we are looking at a brain injury (concussion) that can result in mental health issues, such as anxiety and depression, the underlying biology will be of interest to immunology, neurology, neuroscience, psychology and psychiatry researchers.

In the long-term, researchers investigating treatment options for mTBI will benefit from the understanding of how the immune system contributes to injury and symptoms. Ultimately, this will benefit the patients themselves.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work we will:

Coordinate triannual meetings for our local institutes that cover immunology, neuroscience and barrier biology to update on our findings and receive feedback on the project. This will inform a wide range of immunologists, neuroscientists and clinicians working on brain injury and disease. We will organize biannual meetings with the local Concussion Research Group to disseminate findings to a wider scientific audience, but still within the realm of mTBI.

Disseminate data to a wider scientific audience and foster collaboration, by the traditional routes of national and international scientific conferences and seminar series, and use our active twitter account to highlight our own and others' findings.

All findings will be published on open-access preprint servers (i.e. biorxv), including negative data sets.

Any outputs will be assigned a digital object identifier (DOI) to facilitate sharing and accessibility of them.

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 will be using adult mice to investigate the biology of mild Traumatic Brain Injury (concussion).

Though it is clear that differences exist between mice and human brains in terms of structure, function and geometry, there is still substantial similarity in the physiology, including the immune and inflammatory response. The study of basic biology of mTBI is not possible in humans and raises significant ethical concerns in non-human primates. Lower species than rodents were considered, but due to the mouse brain's similarity to humans, and that measurement of behavioural symptoms is well characterised in mice and is a key part of our proposal, we have chosen this species.

Children and adolescents are also at risk of mTBI, however, the developing brain can be quite different from that of an adult. Therefore, this proposal will focus on the biology of adult mice after mTBI to ultimately understand the response in adult human injury.

Typically, what will be done to an animal used in your project?

Wild type and genetically altered mice will be used to investigate the immune mechanisms that may be important in brain injury. Whilst under general anaesthesia, adult mice will receive a single impact to the head, or two impacts to the head (24 hours apart), referred to as a repeated mTBI (rmTBI), from a controlled electromagnetic impact device. Mice will not undergo any surgical procedures relating to the impact of their heads. Anaesthetised mice will lie, unrestrained on a sponge platform, allowing free movement of the head after impact, to represent the situation in human mTBI. Each mTBI, including induction of anaesthesia takes no longer than five minutes.

The refinement of precise and reproducible impacts will be confirmed prior to the above and therefore implemented as standard throughout the experimental procedures.

Mice will be closely monitored following the procedure and returned to their home cage

after recovering from anaesthetic.

Mice may undergo behavioural testing following sham (a control group that mimics all aspect of the mTBI procedure, except the impact itself), mTBI or rmTBI or naive controls (for up to three months) to assess their neurological, emotional, and cognitive function. The tests are painless, stress-free and non-invasive.

To investigate the underlying biology, in wild type or genetically altered mice, substances may be administered to prior, during or after the mTBI or rmTBI to manipulate the immune response to head injury.

Standard routes of administration will be used, such oral, intravenous or subcutaneous but also direct injection to the central nervous system. This will require general anaesthesia and surgical exposing of skull using aseptic technique. Procedures requiring surgery will only be performed once per animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Mild TBI is typically associated with a brief depression in breathing, followed by a prolonged time to recovery from anaesthetic. Following waking from anaesthetic, animals may appear unwell (self isolate, reduce movement), which typically gets better within 24 hours. After this, animals appear normal. After 24 hours, only sensitive behavioral tests can distinguish animals that have had a mTBI compared to animals that have not.

Our data shows that mice lose no more than 5% body weight 24 hours after, which returns to normal after 2-3 days.

Our previous experience indicates that in a proportion of animals, breathing does not return to normal and the animal does not wake up from anaesthetic (<10% animals).

A rare complication is that mTBI causes fracture of the skull (0.03%).

Injections to the cisterna magna, a fluid filled region at the back of the brain, are performed under anaesthesia, as a result animals have few associated adverse effects. Local bleeding is possible after surgical incision of the skin.

Animals will experience stress or transient discomfort during restraint for injection of substances or inhalation of gaseous anaesthetics (100% incidence).

Expected severity categories and the 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 mTBI, rmTBI and substance injection to the central nervous system is moderate (75%).

Behavioural test are mild severity (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 study of basic biological mechanisms of mTBI is not possible in humans. We aim to understand how the immune system regulates complex behaviours after a brain injury, which can only be done in animals.

Which non-animal alternatives did you consider for use in this project?

Organoids (cells grown in vitro to resembles an organ), also called 'mini-brains' were considered to assess cellular processes, but are not relevant to the study of behaviour.

Why were they not suitable?

Complex behavioural changes cannot be studies in cells, no matter how complex current 'mini-brain' technologies are.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

From analysis of our previous studies and consultation with a statistician and use of the National Centre for the Replacement, Refinement and Reduction of Animals in Research 'Experimental Design Assistant' (NC3R's EDA) has been used to calculate the number of animals needed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Multiple read-outs will be measured in the same experiment, e.g. multiple behavioral tests measuring different aspects of behaviour in the same mice and a full range of tissue taken and analysis performed from the same animal after it is killed.

The NC3R's experimental design assistant has been used, and will continue to be used, to determine the minimum number of animals to provide sufficient power to analyse relevant effects sizes of treatments.

What measures, apart from good experimental design, will you use to optimise the

number of animals you plan to use in your project?

For breeding and maintenance, the number of animals born from genetically altered animals will be reduced by following good colony management strategies described in on the nc3rs website, such as holding stock but not breeding, intermittent breeding when necessary. Behavioural studies will use an optimal number of tests to maximise the information from each animal. The number and order of these tests has been previously validated to ensure each parameter can detect change in behaviour without overstressing the animal.

Pilot studies will be undertaken when starting new experiments to inform experimental design, i.e. power calculations, again optimising animal use.

A continued effort will be made to share tissue from experimental animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

A mouse model of mild Traumatic Brain Injury (mTBI) and repeated mTBI (rmTBI; two successive injuries) (often referred to as concussion) will be performed. Mice will be under general anaesthesia during the head injury and peri-operative pain medication will be administered which will reduce stress and pain for the animal. Mice will not undergo any surgical procedures relating to the impact of their heads.

Mice will be closely monitored following the procedure and quickly returned to their homecage with their littermates after recovering from anaesthetic to reduce stress.

Why can’t you use animals that are less sentient?

Lower species than mice were considered, but due to the mouse brain's similarity to humans, and that measurement of behavioural symptoms is well characterised in mice and is a key part of our proposal, we have chosen this species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In our previous experience, a small proportion of animals (<10%) do not wake from anaesthetic as their breathing does not recover. Although the animals do not suffer, as they continue to be under anaesthetic, we will refine our procedure to increase the accuracy and therefore reproducibility of the impact site. We hypothesise that herniation of the brain stem, due the head being pushed back into the body, rather than moving freely downwards to the sponge bed is responsible for the mortality, which could be a result of misplacement of the impact site on the head. We will therefore create an indentation in the

sponge to hold each mouse in the exact same place and reduce variability of the impact site between animals. Further, we will perform preliminary tests in cadavers to ensure the setup produces consistent impact sites without pushing the head towards the body and without skull fracture, before any live animals will go through the procedure. After these refinements, if any animals do still die after impact, such deaths will be followed up by post mortem investigation to attempt to find the cause of the issue.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All relevant NC3R's guidance and updates will be engaged with, including sign posting to published studies, e.g. FRY, D. 2014. Chapter 8 - Experimental Design: Reduction and Refinement in Studies Using Animals. In: TURNER, K. B. V. (ed.) Laboratory Animal Welfare. Boston: Academic Press and the ARRIVE guidelines for reporting animal research (Kilkennyet al., 2010) - Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improvingbioscience research reporting: the ARRIVE Guidelines for reportinganimal research. PLoS Biol 8:e1000412

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Continued engagement with the NC3R's resources will be undertaken. In particular continued use of the EDA will facilitate regular assessment of the 3R's and recent developments whilst also enhancing experimental design.

Attendance of workshops organised by the animal facility will be undertaken as well as continued consultation with the N3CRs officer and staff at the facility.

115. Influence of gene expression on body plan formation in vertebrate embryogenesis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

segments, skeleton, embryo, somites, Notch

Animal types	Life stages
Mice	embryo, adult, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate how the activities of specific genes control the formation of the basic body plan in early development, using mouse models that remove specific gene functions in tissues of interest at a specific time during 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?

Early stages of body plan formation are highly conserved in all vertebrate species so we can learn how tissue patterning and organ formation occurs during early stages of human development by studying mouse and chick model systems. Moreover, the signals that cells use to communicate with one another during development are signalling pathways that also play a vital role in the patterning or regeneration of adult tissues. When these signalling pathways do not function properly, they can give rise to terrible developmental disorders and also underlie many adult disorders, so the more we understand about how these pathways pattern tissues and interact with one another during organ development,

the better informed we will be as to what goes wrong in diseases where they are not functioning properly.

What outputs do you think you will see at the end of this project?

Peer reviewed publications

Poster presentations and oral presentations at National and International scientific conferences Public engagement communication events
The project scientific outputs will be an improved understanding of the genes involved in body segmentation and how these genes are regulated in normal development. They may also shed light on the causes of some developmental disorders.

Who or what will benefit from these outputs, and how?

Scientists working in the fields of developmental biology and cell signalling will benefit from the enhanced understanding of how key steps in early development are executed. We expect in the future that better knowledge of the basic biology could lead to better management of developmental disorders.

How will you look to maximise the outputs of this work?

Collaboration with scientists and mathematic modelling researchers in the UK and further afield. Dissemination of new knowledge through publications and presentations at conferences. Publication of unsuccessful approaches.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

It is difficult to study the intricacies of embryonic development in the human species and without the use of a model organism. However, much of the knowledge obtained from research carried out in other vertebrate species, such as chick and mouse, can be extrapolated to human embryo development and contributes to the understanding of the basis for many pathological conditions. The main process we study in the lab is segmentation - the body of all vertebrates is made up of segments or units, seen most overtly in the skeleton which is made up of a series of bones. In humans, defects in segmentation can lead to congenital scoliosis (CS), (although we don't yet know how), where babies are born with an abnormally curved spine, a disease with an infant mortality rate of 50%. Defects in embryo formation can also cause other vertebral skeletal diseases including the family of spondylocostal dysostoses (SCD), where babies are born with abnormal spines and ribs, causing the chest to be too short and meaning that they will have breathing difficulties.

It is necessary to study the interplay of these genetic networks in an intact living complex

system because we have no way to generate anything like this in the lab. The process we are studying can be analysed at very early stages of development. At this timepoint these processes are very similar across all vertebrates and we are able to study chicken and mouse embryos as a model for humans as we know that they will be developing in the same way that a human embryo would.

We plan to do studies in mouse and chicken embryos at stages so early in development that the procedures will not be regulated under ASPA. However, we do need to generate pregnant genetically altered mice and, in some cases, to administer substances that will control the activity of specific genes in the embryos that they are carrying.

Typically, what will be done to an animal used in your project?

We use normal fertilised chick eggs, so all procedures relating to this species fall outside regulation under ASPA.

In the mouse studies, we will breed genetically altered animals. Pregnant females will be killed humanely at early stages of embryo development and we will recover the embryos for detailed analysis in the laboratory. In some cases we will deliver a substance (usually tamoxifen, used in people to treat some types of cancer) to "turn on" the gene of interest. The tamoxifen will be given by gavage (where a tube is gently passed through the mouth into the gullet of the pregnant animal), a couple of days before she is humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Mouse breeding is not expected to be associated with any detectable clinical signs; the animals will look and act like normal mice. Tamoxifen administration can cause the pregnant mice to lose some weight and eat less (relatively common), and there could be a low level of abortion (not seen by us to date). In the very unlikely event that any animal that appears seriously unwell, it will be humanely killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The genetically altered mouse lines bred are expected to look and act completely normally and have no clinical signs of any type, and so are all expected to have “sub-threshold” severity. As described above, the Tamoxifen may make the pregnant female mice feel briefly unwell and the dosing procedure can cause temporary mild discomfort. We therefore expect most of the females dosed to have a “mild” severity, with a few possibly being “moderate” (<5%), simply because these signs may take a few hours to resolve completely.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you

have 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 difficult to study the intricacies of embryonic development in the human species and without the use of a model organism. However much of the knowledge obtained from research carried out in other vertebrate species, such as chick and mouse, can be extrapolated to human embryo development and contributes to the understanding of the basis for many pathological (disease) conditions. In humans, defects in embryo development (in the process of segmentation) lead to congenital scoliosis (CS), a disease with an infant mortality rate of 50% that comprises many vertebral skeletal pathologies including the family of spondylocostal dysostoses (SCD). For CS the aetiology is still unclear. It is necessary to study the interplay of these genetic networks in an intact in vivo system. When appropriate we will use in vitro systems for molecular and cellular analysis. We will ensure that any new relevant replacement technologies which become available during the life-time of grant will be fully investigated and employed if possible.

Which non-animal alternatives did you consider for use in this project?

We have developed in the lab a protocol where we can add reagents that instruct stem cells (mouse or human) to adopt a cell fate in vitro which has the hallmarks of the cells that make up the tissue that segments to form somites in the embryo. The stem cells thus provide a system we can use to investigate the consequential changes in expression levels of genes and proteins brought about by changing the function of a gene of interest in the process we are studying: namely segmentation. However, this is an assay or protocol that monitors changes in cells in culture which do not form 3d structures as found in the embryo. In order to understand what effects the change in gene function may have to the shape or timing of formation of the 3d segmental structures of the somite in a temporal and spatial context it is essential to have the 3 d animal model.

Why were they not suitable?

in order to study the effect of how genes work in relation to the various structures of the embryo over time gene it is essential to have the 3D animal model - this cannot be addressed using a cell culture model because the embryo is changing all the time and how the changes all interact is important in how the gene is working at any one time

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We need to look at how genes from pathways of interest work in the tissues of interest in the developing embryo during segmentation using different methods. These methods include looking at the expression levels of RNA and proteins encoded by the genes of interest and how these expression levels change in transgenic animals where the expression and thus function of key signalling pathways have been compromised. In a typical year, we will use 60 informative mutant embryos per line of genetically altered mice

for analysis of RNA expression, and 20 informative mutant embryos per line for protein expression analysis. For the three genetically altered lines we might be working on at any one time, we estimate that we will need about 200 adult animals per annum, or 1000 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 have established the smallest possible breeding programme that will still provide embryos of 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?

We aim to extract as much information as possible from each embryo, so as to minimise the total number of embryos that we need to produce.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 our work is carried out under conditions that do not require a licence. Our main animal model is the chick embryo and all experiments are terminated prior to halfway through embryonic development and so are not regulated under ASPA. We will only use the mouse knockout model to answer specific questions that cannot be satisfactorily addressed in the chick system. We will breed genetically altered mice in such a way that they don’t show any detectable clinical changes. We do this by making sure that, where the gene of interest being changed might cause harms, one of the two copies is normal (heterozygosity) or that it is only turned on briefly when we need it (using Tamoxifen to do this as a “switch”). The only procedure that the animals will be aware of is the dosing of Tamoxifen (in a small number of mice). We use gavage rather than injection because this is considered less harmful for the mice. All experimental work is done on tissues from embryos that are considered not to be sentient and, for the mice, these will be harvested after the mothers after have been humanely killed.

Why can’t you use animals that are less sentient?

We will be using mouse and chick embryos that are not sentient as they are earlier than half way through gestation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We shall ensure the welfare of the adult breeding animals is our priority and we will

simultaneously ensure we establish the smallest possible breeding programme that will still provide embryos of the required genotype.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will have regular discussions with the Named Persons and animal technicians at my institution to review current approaches and whether there are any new 3Rs opportunities. I will subscribe to the NC3Rs e-newsletter, attend NC3Rs events and workshops, register for upcoming webinars

Home Office

116. Gene Therapy for Glaucoma

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Glaucoma, Gene Therapy, Blindness

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 viral gene therapy for Glaucoma.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Glaucoma is a disease that causes increased pressure within the eyeball. Left untreated the disease results in progressive irreversible blindness due to damage to the optic nerve and key retinal cells.
Glaucoma is the leading cause of blindness worldwide and is estimated to affect over 11 million people. In the developed world the condition affects around 2% of people over the age of 40. Multiple clinical trials have shown that long-term reduction in intra-ocular pressure is effective at preventing the visual loss associated with glaucoma.

Currently, the treatment of glaucoma is by daily administration of eyedrops. Treatment

needs to be continued for life and patients require regular monitoring to ensure their dosing regime remains at the correct level. As a result, treatment is expensive and in the US is estimated to exceed $3 billion per year. Treatment failure occurs commonly and is invariably linked to poor patient compliance, often driven by the side effects of the drugs used in treatment. Consequently, there is an urgent need to develop more effective treatments.

What outputs do you think you will see at the end of this project?

The outlined studies are expected to generate the data needed to advance a novel treatment for glaucoma through proof of principle towards first in human clinical trials.

Who or what will benefit from these outputs, and how?

The short-term beneficiaries of this work will be the scientists involved in the study and will be in the form of employment and career development. In the medium term, the data generated will be of benefit to fellow scientists working to develop novel gene therapy treatments for diseases including a range of ocular diseases. For glaucoma, it will permit identification of optimal targets and refinement of the delivery systems for this type of treatment, which aims to target specific genes involved in the development and progression of disease. In the long term, and following safety testing in larger animals, the study is expected to lead to the development of a new and more effective treatment for glaucoma which will be of benefit to both patients and health care providers.

How will you look to maximise the outputs of this work?

The outputs of the study will be maximised through collaboration with other scientists working in relevant disciplines. In addition, much of the data generated will be presented at scientific conferences, published in peer review journals and made available through open access repositories.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used for these studies as they are the least sentient of the species for which established models of glaucoma exist. Adult mice will be used as the techniques for imaging and assessment of intraocular pressure, required to monitor the disease, are easier to perform at this stage of development.

Typically, what will be done to an animal used in your project?

Typically, mice will be anaesthetised and given a single injection of micro-beads into the front chamber of the eye to induce glaucoma. Subsequently, the mice will be given a viral vector by injection into the eye performed under general anaesthesia. Mice undergoing

these procedures are not expected to show any signs of pain or discomfort upon recovery from anaesthesia. The development of glaucoma is non painful and whilst it may cause some visual impairment this is not expected to adversely affect the behaviour of the mice.

During the study, the intra-ocular pressure of the eyes will be measured, using a painless method, up to twice weekly. Further imaging may be undertaken on up to 10 occasions under general anaesthesia, at not more than once weekly intervals. At the end of the study the mice will be killed using a humane method.

What are the expected impacts and/or adverse effects for the animals during your project?

All procedures likely to cause pain will be conducted under general anaesthesia. In all cases, the mice are expected to make a rapid and uneventful recovery from anaesthesia. The disease model used is non-painful and, whilst it may cause some visual impairment, is not expected to adversely affect the general wellbeing of the mice as they are nocturnal and therefore do not rely heavily on vision when moving around the cage or seeking food and water.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for all animals is 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?

It is not possible to evaluate the efficacy of novel treatments for glaucoma without the use of living animals as intra ocular pressure, which is elevated in glaucoma and is ultimately responsible for causing visual loss, is only maintained while the animal is alive and the eye has an intact blood circulation. It is also essential for the translation of novel treatments into clinical trials that effectiveness is first demonstrated using an accepted animal model.

Which non-animal alternatives did you consider for use in this project?

A range of laboratory-based studies will underpin the outlined animal experiments. These include the use of established cell lines and primary cell cultures to identify and assess target genes and assess viral vectors. In addition, to assess whether a therapeutic modality can translate into man, studies will also be undertaken using human eye tissue from post-mortem donors when possible. Only vectors shown to be effective at suppressing gene expression in laboratory-based studies will be progressed into animal

studies.

Why were they not suitable?

Whilst the successful outcome of laboratory-based studies will be a prerequisite for all animal experiments, it is only by using an animal model of glaucoma that the actual effectiveness of the vector at reducing intra-ocular pressure can be assessed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of the number of animals required is based on the research group's experience in undertaking related studies and takes into account pre-existing knowledge of the planned number of relevant molecular pathways and potential therapeutic targets that we have identified for down regulating intra-ocular pressure, together with the available expertise for developing viral vectors.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Online tools were consulted however, these assume the use of parametric statistics, which is inappropriate for the assessment of clinical data generated by rodent models of intraocular pressure, even after standard normalisation procedures. Consequently, statistical packages in R that allowed estimation of sample sizes based on data from previous work were used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The outlined gene therapy will be given into one eye only, thereby enabling the contralateral eye of each animal to be used as the non-treated control. In so doing, each animal acts as its own control, thereby minimizing biological variation and consequently the number of animals required to complete the study. In terms of intraocular pressure this is acceptable as eyes are sufficiently independent.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm

to the animals.

The outlined microbead model reliably induces raised intraocular pressure to replicate glaucoma disease as it occurs in the human eye. This model induces a mild disease state, which is not painful, and the associated loss of sight does not impair the animal's ability to perform its normal behaviour. The induction of the model and the delivery of the gene therapy, both of which involve giving an injection into the eye, will be performed under general anaesthesia. The response to treatment will be determined by monitoring intraocular pressure non-invasively using a painless rebound tonometer, which can be performed on the animal while it is conscious without causing distress. In addition, non-invasive imaging of the retina will be conducted under general anaesthesia at appropriate intervals throughout the study.

Why can't you use animals that are less sentient?

Mice are the least sentient of the animal species that have eyes that are anatomically similar to humans and for which established models of glaucoma exist.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures used in the outlined studies have been extensively refined and the animals are not expected to show any overt signs of suffering as a result of the procedures undertaken. To ensure the methods used remain the most refined, the scientists involved will attend virtual workshops with other groups specialising in glaucoma research to share knowledge and experience.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The outlined studies will be undertaken in line with GLP. The injection techniques used will be in accordance with the guidelines published by the NC3Rs and LASA.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stay abreast of 3Rs developments through continuing education including attending and contributing to 3Rs focused seminars and lectures. In addition, I am regularly updated on 3Rs development by our regional NC3Rs programme manager, and through my contacts with members of the university's AWERB and Animal Services Unit. I am also involved in a networking group linking researchers working on glaucoma and regularly attend international conferences such as Association for Research in Vision and Ophthalmology (ARVO).

117. Investigating the Role of Molecular Danger Signals in the Pathogenesis and Resolution of Gut Inflammation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Inflammatory Bowel Disease, Inflammation, Immunology, Damage associated molecular patterns, Colitis

Animal types	Life stages
Mice	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?

To understand how molecular danger signals initiate and maintain gut inflammation, and the subsequent biological processes that improve and resolve gut inflammation in Ulcerative Colitis and Crohn's disease (the Inflammatory Bowel Diseases, IBD).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

IBD is an incurable chronic immune-mediated condition affecting the gastrointestinal tract.

In UK, IBD affects 1 in 125 individuals. Worldwide, the global prevalence is projected to affect 25-30 million individuals by 2025.

IBD is characterised by a persistent and non-resolving inflammation that damages the gut lining.

IBD sufferers typically experience marked symptoms such as uncontrollable bowel habit, significant bloody diarrhoea, abdominal discomfort, malnutrition and potentially, complications relating to bowel obstruction, perforation or infection.

Current treatments typically block the immune response; they are long-term, expensive and are associated with side-effects in relation to immunesuppression. They do not cure IBD and patients are susceptible to recurrence of IBD flare-ups.

These difficulties in clinical management and treatment are mainly due to our fundamental lack of understanding of the molecular events that lead to the development of IBD.

The program of work outlined in this PPL proposal seeks to address how gut 'danger signals' drive the development of gut inflammation.

What outputs do you think you will see at the end of this project?

Three major outputs:

The identification of new disease-specific pathways and mechanisms in IBD

Discovery of new drug targets that will allow further pre-clinical development

Discovery biomarkers of disease mechanisms and inflammation that can be used in the human setting in the clinic

Who or what will benefit from these outputs, and how?

Allied scientists in gut biology and immunology (Short-term 1-5 years)

We are working in a multi-disciplinary manner with gut immunologists, biologists, clinician-scientists, chemists and data scientists to assimilate our understanding of inflammation. The use of mouse models of colitis in this proposal is central.

Current work involves how T-cells, dendritic cells, macrophage and neutrophils (components of the adaptive and innate immune system) interact with gut danger signals vis-a-vis microbiota and epithelial barrier to drive the inflammatory process in inflammatory bowel disease (IBD).

Allied researchers in drug development and clinical translation (Short-term 1-5 years)

1. The identification of novel drug targets, pathways or biomarkers in relation to the effect of gut danger signals can be verified, validated and further tested using the mouse models of colitis as described in this proposal.

Clinicians in IBD (Medium term 3-7 years)

The pre-clinical discovery of drug targets and biomarkers can be translated into novel drugs or biomarker test that can be used in the clinical setting.

Existing approved drugs that target the gut danger signals as implicated in our pre-clinical work can be 'repurposed' and tested in human clinical trials to determine efficacy in IBD.

Patients with IBD (Medium-to-long term; 3-10 years)

The pre-clinical discovery of drug targets and biomarkers can be translated into novel drugs or biomarker test that can be used in the clinical setting.

Existing approved drugs that target the gut danger signals as implicated in our pre-clinical work can be 'repurposed' and tested in human clinical trials to determine efficacy in IBD.

Both (1) and (2) can lead to direct benefit of patients with IBD and may lead to better understanding of IBD with a potential to finding a cure.

How will you look to maximise the outputs of this work?

Collaboration:
We are working in a multi-disciplinary manner with gut immunologists, biologists, clinician-scientists, chemists and data scientists to assimilate our understanding of IBD-inflammation. The use of mouse models of colitis in this proposal is central. In this context, there >15 Principal Investigators in our group working on IBD from various angles and aims - both all with the purpose to deliver better treatments and understanding in IBD.

Dissemination of new knowledge:

We have several dynamic channels including within our university and regional and national resources to allow us to rapidly disseminate and share our new knowledge pertinent to this PPL work.

Publications:

We aim to publish all findings and where new techniques or difficulties are encountered, these knowledge will also be shared in the form of methods publication.

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.

Live animals (mice) are necessary because of the complex interaction between luminal bacterial components, the mucosal and systemic innate and acquired immune systems and critically, the mucosal barrier.

The mouse model is a very well-described and extensively used approach to recapitulate

the complex gastrointestinal system, especially in the study of IBD.

There is not an adequate in vitro system that closely recapitulates this complex physiological make-up. Furthermore, almost all immortalised human intestinal epithelial cell lines are derived from colorectal cancer. As demonstrated, whenever possible, we have performed our studies in vitro, but final proof of our hypothesis needs to be tested in living systems in this setting.

The mouse gastrointestinal biology closely resembles the human with a well-documented immune system and microbiome that can be compared to the humans and further analysed if they share high level of homology. Furthermore, both the genetics and microbiome in the adult mouse system can be manipulated to address our scientific questions.

Typically, what will be done to an animal used in your project?

Typically, there are two types of settings.

Use of mouse as the model for gut inflammation:

Gut inflammation will be directly induced acutely (within 5-10 days), in a chronic manner (over 4- weeks) or the susceptibility to colitis increased (i.e. by using susceptible strains in certain conditions).

Induction of colitis maybe induced by direct oral toxin (e.g. dextran sulphate sodium in drinking water), by rectal instillation (e.g. oxazolone); or susceptibility induced by gene-silencing/knock-out (e.g. tamoxifen injection in Cre-mediated gene excision) or transfer of susceptible mice into specific- pathogen free conditions.

In acute colitis, experiments usually usually conclude within 7-10 days.

In chronic colitis, 2-4 weeks.

Susceptible strains maybe maintained up to 3 months to observe for signs of colitis (e.g. diarrhoea).

At the end of experiment, all mice are humanely culled and the guts are harvested for experimental analysis.

Use of mouse to study relevant pathways and drug targets pertinent to IBD:
Our experimental mouse might be given a pharmacologic agent to treat or modify inflammation (by oral, injection or rectal instillation).

All mice within an experiment are used once and humanely culled at the end.

Some may require injection of agents prior or during the induction of colitis.

What are the expected impacts and/or adverse effects for the animals during your project?

In colitis:
Onset of diarrhoea, rectal bleeding and weight loss.

In acute models, onset can be within 3-4 days; and in chronic models, within 7-10 days.

Mice may display signs of general unwellness e.g. hunched or visually withdrawn.

In all experiments, all mice are monitored on a daily basis using well-established colitis scoring models to ensure that all mice that display signs of severe colitis are humanely culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severity: Mild-to-moderate 40% Mild vs. 60% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Live animals are necessary because of the complex interaction between luminal bacterial components, the mucosal and systemic innate and acquired immune systems and critically, the mucosal barrier.

The use of the established mouse models of gut inflammation allows us to study all the key stages of inflammation from the onset, the peak phase and the resolution of gut inflammation.

This also allows the manipulation of genes, different immune cells (either those present in the gut all the time vs. those that are activated or recruited to the gut during inflammation) and the potential of modifying inflammation using potential pharmacologic approaches.

Which non-animal alternatives did you consider for use in this project?

We considered gut cell lines and/or primary explant cultures directly from humans.

Why were they not suitable?

There is not an adequate in vitro system that closely recapitulates this complex physiological make-up which involves interactions with the gut immune cells, bacteria and the lining of the gut.

Furthermore, almost all immortalised human intestinal epithelial cell lines are derived from colorectal cancer. As demonstrated, whenever possible, we have performed our studies in

vitro, but final proof of our hypothesis needs to be tested in living systems in this 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?

Yes. We carry out statistical calculations to ensure that the lowest numbers of mice are used in our experiments to get the data needed.

We will carry out pilot studies and consider the most efficient breeding methods to minimise the number of mice used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experimental plans are clearly written, reviewed (internally and by our vets) and in accordance to our protocol before implementation.

We review the statistical numbers and also the current literature for our experimental plans.

We clearly defined our experimental goals so that the maximum data can be obtained to reduce need for further 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?
Where possible, we will share the colitis tissue with relevant collaborators to reduce number of animals used.

We routinely share our planned experiments and shared protocols with our internal collaborators to ensure that our experimental animals can be shared where possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the mouse model of IBD, which is established and well-described for at least

30 years. We have 10 years of experience (the applicant is a PPL holder since 2010). Our models used, and with benefit of primary experience - we will ensure that our methods are associated with the least form of distress and suffering.

These models and methods are chosen - most with a short duration of colitis (DSS/TNBS/oxazolone) or insidious and mild colitis (piroxicam/mitochondrial stress inducers) that can be accurately monitored to allow the least amount of suffering.

Why can’t you use animals that are less sentient?

The optimum stage to induce colitis in the mouse model relies on the mature development of the gut bacteria, physiology and immunology. Hence, earlier life stages are not appropriate.

The gut dies rapidly in the event of terminal anaesthesia, hence this is also considered as not appropriate to our research questions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have developed a Colitis Monitoring Score over the last 10 years which we have successfully used to identify and ensure that mice are humanely culled as soon as they breach the severity threshold.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are routinely engaged with all the Rodent Animal Users and Gut Inflammation groups in UK to ensure that we are up to date with the best practice guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our group will regularly attend all the seminars and training courses offered by our local Veterinary Services.

We will make sure that we implement all advances of the 3Rs with all department's support.

118. Understanding mechanisms for controlling picornavirus infection

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Picornavirus, Conserved Epitopes, Protective Immune Responses, Antivirals, Disease Resistant Animals

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?

Viruses in the picornavirus family include important pathogens of animals and humans. The overall aim of this project is to use mouse models of infection to understand the replication of these viruses and to develop novel approaches to control the diseases they cause.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 animal picornavirus relevant to this application is foot-and-mouth disease virus (FMDV), which is responsible for one of the most significant diseases of livestock, leading to large economic losses due to reduced productivity and trade embargoes for areas not certified as disease-free. The disease is endemic to many low-income countries,

compromising economic and social development and contributing to poverty and poor nutrition in rural communities. Occasional outbreaks of FMD also have a significant impact in high-income regions in which the disease is not normally present, with the UK outbreak in 2001 leading to losses of ~£10 billion. Vaccination can be used to control FMD but current vaccines have notable shortcomings, such as short duration of immunity and poor protection against diverse circulating strains. In order to overcome these shortcomings, there is a requirement to understand how to induce more broadly protective and longer lasting immune responses. An alternative strategy to prevent FMD in livestock is to genetically modify the animals so that they are no longer susceptible to the virus. The concept of such genetic modification (GM) has been significantly refined in recent years by a process known as gene editing where advantageous traits can now be introduced with very subtle changes to the animal genetics and without insertion of any foreign genetic material.
The work in this project is required in order to assess the suitability of such technology for generating FMD resistant animals.

Human pathogens in the picornavirus family are exemplified by viruses in the enterovirus genus of the family which includes human rhinovirus (HRV). HRV is thought to infect humans more frequently than any other virus, being responsible for approximately 70% of all respiratory tract infections (the common cold) and was estimated in 2003 to cost $40 billion annually in healthcare and lost productivity in the US alone. In the last two decades it has been recognised that HRV infection is also associated with more serious clinical outcomes such as severe lower respiratory tract infections of infants and exacerbations of chronic lung diseases (asthma, cystic fibrosis and chronic obstructive pulmonary disease). HRV therefore creates an enormous direct and indirect social and economic global burden. There are no vaccines or drugs available to control or treat HRV. HRV is the main target enterovirus for this work but the approaches proposed would also be of relevance to a number of other important and closely related viruses in the family. For example, a number of enteroviruses have emerged in the last decade to also become significant concerns for regional and global public health, such as enterovirus 71 (EV71), enterovirus 68 (EV68) and Coxsackieviruses which are responsible for large epidemics in China, South East Asia and the US and which have featured severe disease including neurological complications and child deaths. The work in this project will contribute to novel antiviral or vaccine approaches to control these viruses.

What outputs do you think you will see at the end of this project?

At the end of this project we will have gained new understanding of i) the relationship between virus replication, pathogenesis and immune responses, ii) the anti-viral effect of antibodies capable of recognising all variants of a virus, iii) antigen persistence and its role in enhancing antibody responses and iv) novel antiviral approaches and host genetic targets for engineering disease resistant animals. All the outputs will be communicated by publication in high quality peer reviewed, open access scientific journals.

Who or what will benefit from these outputs, and how?

In the short-medium term, the new knowledge derived from this work will have academic impact from improved understanding of virus replication and antigenicity. Information relating to antibody responses will be translated to further studies in the natural target species for FMDV, in order to develop improved vaccines to control FMD. Gene targets found to be suitable for editing for engineering disease resistant animals will be translated to studies to make gene edited pigs resistant to FMD. Novel approaches for vaccines and

antivirals against HRV or other human viruses identified in this work may lead to human clinical trials. In the long term this work could contribute to improved vaccines against FMDV, the development of FMD resistant pigs and other livestock, novel vaccines and antivirals for HRV and other human picornaviruses.

How will you look to maximise the outputs of this work?

The outputs of this work will be instrumental in guiding i) strategic research to improve FMD control within the establishment, ii) existing collaborative studies aimed at generating disease resistant animals, iii) existing collaborative studies to investigate novel vaccines for HRV. Knowledge will be further disseminated through scientific meetings and networks such as the Global foot-and-mouth disease Research Alliance (GFRA) the Food and Agriculture Organisation (FAO) of the United Nations, the European Study Group for the Molecular Biology of Picornaviruses (EUROPIC) and medical networks via clinical collaborators.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used for these studies because the use of adult mice as models for studies of immunogenicity and virus infection are well established, such that experiments can be designed with predictable outcomes that minimize pain, suffering, or distress. Inbred mice will be used to ensure uniformity between all animals, increasing the predictability of experiments and reducing the number of animals required. The use of mice also provides access to existing collections of gene knock out animals which are readily available from UK and global repositories.

In addition, measures for refinement of experimental conditions such as enhanced environment can be implemented effectively.

Typically, what will be done to an animal used in your project?

A typical animal might experience up to four injections of immunizing antigen over several weeks and/or injection to inoculate with virus, which may result in infection lasting up to one week. Small volumes of blood sampled periodically throughout.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of effects will be mild. For example, animals will experience mild and transient pain associated with an injection or blood sample being taken. Some infected animals may experience discomfort and distress, weight loss or abnormal behaviour due to the onset of disease which may reach a moderate level for a short period.

After intranasal inoculation of adult mice with HRV, viral replication is restricted to relatively low viral loads in the airways, causing mild signs of infection, typically transient weight loss, ruffled fur and lethargy, with recovery within 7 days.

Intranasal inoculation with enterovirus-68 (EV-68) in mice also produces an acute disease with signs of infection including transient weight loss and recovery within 7 days.

FMDV causes an acute infection in adult mice after intraperitoneal injection. Disease severity can range from subclinical to lethal and is determined by the virus strain used. Strains of FMDV which reproducibly induce either mild or severe disease have been previously characterised. Low- pathogenicity strains of virus result in mild, transient clinical signs such as weight loss, ruffled fur and lethargy (also measurable virus in the blood) which usually resolve within 7 days. Pathogenic strains of virus can lead to systemic infection with virus replicating in multiple organs leading to weight loss, respiratory distress, neurological signs and death. Where possible, virus strains leading to only mild disease will be used but in some studies more virulent strains may be required. However, in all cases, severity will be limited by careful monitoring and humanely killing any animals reaching moderate severity limits.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals (80%) will experience only mild effects. The remainder (20%) are predicted 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 carry out research to understand and control viral diseases of animals and people. After many years of in vitro research (not using animals) we have developed several new strategies that we believe will help us understand how to make better vaccines or allow us to produce livestock animals that are resistant to disease. The only way for us to confirm if these strategies will work is by doing experiments with animals.

Which non-animal alternatives did you consider for use in this project?

In vitro methods such as cell culture have been used extensively in the research leading to this application. Primary cell cultures, organ on a chip and organoid approaches have been considered for this project. The use of antibody-like reagents generated by recombinant methods may be used instead of generating monoclonal antibodies from animals.

Why were they not suitable?

Cell culture methods will continue to be used wherever possible (e.g. to provide preliminary characterization of attenuated viruses) but no in vitro approach is able to provide assessment of disease pathogenesis or the role of complex immune responses in protecting against infection.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used in these studies has been determined with the help of statisticians to ensure the data generated is scientifically robust, reproducible and uses the fewest animals possible.

Pilot experiments with small numbers of animal numbers will inform and refine the experimental design of subsequent comparable studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In addition to online tools, experimental design has incorporated advice from local statistician, NVS and local researchers experienced in use of mouse models.

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 conducted where appropriate.

The use of mice as models for immunization and for picornavirus infection are well established and outcomes are predictable.

The design of experiments will continue to be scrutinized as the project progresses to optimise the numbers of animals being 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 use of adult mice as models for studies of immunogenicity and virus infection is well established, such that experiments can be designed with predictable outcomes that minimize pain, suffering, or distress.

In addition, mice are thought to be the least sentient mammalian laboratory animal, such that measures for refinement of experimental conditions such as enhanced environment can be implemented effectively.

Why can’t you use animals that are less sentient?

Immature mice or non-mammalian species would not have a relevant immune system response and/or would not be susceptible to infection.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be acclimatized to their accommodation for 7 days prior to the start of any experiment and during this time will be familiarised to handling.

HRV and EV-68 cause mild signs of infection in mice after intranasal challenge, typically transient weight loss, with recovery within 7 days. FMDV causes an acute infection in mice after intraperitoneal injection with disease severity determined by the virus strain and ranging from subclinical to lethal.

Signs of mild disease usually resolve within the first 7 days post challenge and any clinical signs are expected to occur within this period. Therefore, in order to monitor the occurrence of signs and limit the severity, mice infected with pathogenic strains of virus will be observed at least 3 times a day during the first 7 days post challenge time period and weighed daily at the same time each day over this period. If any of the severity limits defined in the protocol are reached, the animal will be humanely culled.

Models of infection will be chosen so that the least severe option will always be selected, for example using infection with less pathogenic strains of FMDV where appropriate and using non-infectious antigen in studies of persistence which will avoid animals being infected.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Refinement best practice specific for mice will follow guidelines issued by LASA and NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Up to date information will be checked via online resources from LASA and NC3Rs and via regular discussion with the local NACWO with special expertise in small animals.

119. Developing models of retinal degenerative disease and trialling drug and gene-based 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Vision, Age-related Macular Degeneration, Gene-Therapy, Laser, Ophthalmology

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 demonstrate the efficacy of drug and gene treatments for retinal degeneration, using models both widely available and being developed in our laboratory.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Age-related macular degeneration (AMD) is the main cause of blindness in elderly people in the developed world. About 1 in 3 people aged over 75 are affected. Patients suffer from a slow degeneration of the light sensitive cells in their retina called photoreceptors and the underlying supportive cells called the retinal pigment epithelium

There are two types of AMD: “Wet” AMD is when “leaky” blood vessels grow through the retina. There are invasive treatments but there is no cure. “Dry” AMD is when the pigmented cells that support the light sensitive cells die. There is no cure and no treatments available.

There are very few good models of Dry AMD. We have developed a mouse model using a laser to treat sections of the retina. Our model shows many similarities to Dry AMD. We believe it will help us to understand exactly what leads to the death of the retinal cells and the loss of vision. We can also use it to try out different treatments.

There is already a good laser model for Wet AMD which we will use to test gene therapies. The current treatment for Wet AMD needs regular injections into the eye. This is unpleasant, expensive, and time consuming. The hope is that with a better understanding of what causes the disease, we can find a gene therapy target that can stop and heal the damage done to the retina.

What outputs do you think you will see at the end of this project?

We are developing a laser model of Dry AMD in our lab which will aid in the research into treatments of this disease. Current models are either genetic models or require the injection of damaging substances into the eye, while the laser model will be non-invasive and reproducible. The development of new models provides platforms for the discovery of novel therapeutic targets.

We hope to use this model to trial new therapies and drug targets for a disease currently without treatments.

We plan to assess the suitability and efficacy of new gene-therapies for Wet AMD, with the hope to take them forward into clinical trials as less painful and repetitive treatments.

Who or what will benefit from these outputs, and how?

Age-related macular degeneration (AMD), a progressive retinal disease that results in the loss of central vision, is predicted to affect 288 million people worldwide by 2040 (Wong et al., 2014).

The consequences of this condition for vision can be severe. AMD is the most common cause of visual impairment in the developed world, and the Royal National Institute of Blind People (RNIB) reports that AMD is the most common cause of certification for vision impairment. The prevalence of late AMD in the UK among people aged 50 years or over is 2.4% (from a meta-analysis applied to UK 2007–09 population data). This increases to 4.8% in people aged 65 years or over, and 12.2% in people aged 80 years or over. The same study found the prevalence of geographic atrophy to be 1.3 to 6.7%, and the prevalence of neovascular AMD to be 1.2 to 6.3%.

Estimates indicate that around 39,800 people develop neovascular AMD in the UK each year; given a total UK population of 60 million, this equates to 663 new cases per million per year. The cost of aflibercept and ranibizumab, medicines for the treatment of late AMD (wet active), is significant. In 2015/16, ranibizumab was second and aflibercept was fourth in the list of medicines with positive NICE technology appraisals on which the NHS spent most money. Between them, they accounted for a total of around £450 million expended (although some of these costs relate to use for other licensed indications).

[https://www.nice.org.uk/guidance/ng82/chapter/Context]

The characterisation of our laser model of Dry AMD will make it a better understood model of AMD, making it more useful for treatment experiments. This will be of benefit in many laboratories around the world who will be able to use our model as it is easy to reproduce.

We expect that we will gain a greater understanding of the molecular pathways of the complement pathways and how they exacerbate the degenerative phenotype of Dry AMD. This understanding will lead to novel treatment opportunities.

Our Wet AMD gene-therapy experiments will provide proof of concept for a variety of gene therapies. This should allow us to provide data to regulators and allow progression to phase 1 clinical trials.

Ultimately, in the long term our work should lead to novel treatments for both dry and wet AMD. This will help the many millions of patients affected with these conditions around the world.

We may collaborate with companies or other academic researchers to test their molecules in our mouse models of Dry and Wet AMD

As a consequence of our previous research in this area three pharma companies are currently interested in collaborating with us to evaluate if their lead molecules for treating AMD show an effect in our model of AMD. Due to confidentiality names are withheld.

How will you look to maximise the outputs of this work?

As we have done previously all results will be published in peer reviewed journals and may be used for conference presentations.

We will seek to move the results from these experiments to phase 1 human clinical trials.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We will use mice as there is a large range of models available. There are some differences in the eyes of mice and humans, but they are very similar in how they work. The way that the light sensitive structure at the back of the eye (the retina) is damaged by disease is very similar. There are models of this damage that have been used by lots of people and are known to work well. We will also be using a model that we have created ourselves.

We begin the experiments when the mice are adult, which is 3 months old. We then watch them over several months to see how aging affects them.

Typically, what will be done to an animal used in your project?

Firstly, the mice will undergo all the measurements to be used later in the experiment. These measurements will be used as normal to compare with the changes as a result of the experiment. They may also have blood taken.

These measurements may include: fundoscopy (imaging the retina - the light sensitive back of the eye), fluorescein angiography (using a fluorescent drug to show the blood vessels of the retina), electroretinography (dark adapted mice are exposed to a bright flash of light, and the electrical responses are measured), optical coherence tomography (imaging the layers of the retina), and testing the vision of the mice by how they react to patterns of moving lines.

For some experiments the mice will be given treatment one week before they are given the laser treatment. This may be an injection into the eye, or it may be eye drops. For other experiments they will be given treatment one week after the laser treatment.

Depending on which form of the disease being investigated the mice will be given one of two patterns of laser to the retina. Age related Macular Degeneration (AMD) has two forms Wet and Dry. For Wet AMD we use separated spots of high intensity laser which breaks Bruch's membrane under the retina and allows blood vessels to grow through. For Dry AMD we use connected spots of low intensity laser which damages the light sensitive cells but doesn't damage the Bruch's membrane underneath.

The laser treatment for the Wet AMD model may be repeated after a month to investigate how long the treatment lasts.

After that they will be followed for several weeks or months with measurements taken no more than once a week. At the final time-point the mice will be humanely killed using either a Schedule 1 method or an overdose of anaesthetic followed by perfusion.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of what we do to the mice is relatively harmless and non-invasive. Normally we would only see a maximum of very mild pain or distress for very short periods of time. By lasering the retina, we are reducing their ability to see. But mice use smell and hearing to interact with the world more than sight and show no distress when their sight is damaged. We are only treating one eye per mouse so they will still have normal sight in their untreated eye.

Rarely the laser treatment to the retina can cause the mice pain. This is usually a sign that something has gone wrong with the experiment. Mice in this situation will be given painkillers while the problem is investigated. If the problem cannot be treated the animals will be humanely killed.

When we give fluorescein to look at the blood vessels in the retina sometimes it can take longer than normal to leave the body. This usually happens in older or weaker mice as their kidneys don't work as well. The fluorescein staying in the system can lead to severe dehydration and in serious cases, death. To help prevent this we have a low maximum dose and with new types of mice we will do a short experiment to find the lowest dose possible that still gets clear results. We may also provide extra hydration for the mice shortly after the experiment by injecting them with saline and giving them water- soaked food when necessary. If the mice are weak and still yellow after a day, they will likely die

and so we will humanely kill them at this point to relieve their suffering.

There can be some health problems when repeatedly anaesthetising mice. They can become more sensitive to the drug or become lethargic. We have limited how many times the mice can be anaesthetised and how much time there must be between sessions. We also use a maximum of 80% of the normal dose in all but the most invasive sessions - like the ones with the laser.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

There are some potential health problems with repeatedly anaesthetising mice. They can become more sensitive to the drug or become lethargic. While we have limited how many times the mice can be anaesthetised and how much time there must be between sessions, there is still a potential problem that requires careful monitoring, especially towards the end of the experiment.

While each individual procedure is unlikely to cause much pain or distress and we put several of them together to reduce the number of required sessions, the sum of anaesthetic treatments over time and the associated stress, means we still expect many of mice to reach the license limit 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?

This work is looking for treatments for disease and we cannot do these experiments on people. We need to be able to see the effects on the whole retina and any side effects in the whole body. We cannot do that with donated tissue and there is no other modelling of the whole retina to allow us to do these experiments without animals. In summary our work must be done in vivo since it is too complex for in vitro/silico work which does not adequately recapitulate the human disease. It would be unethical to use experimental treatments on human patients or to damage the eyes of humans; therefore, our only option is the use of animals, with mouse being the best surrogate of lowest neurophysiological sensitivity.

Which non-animal alternatives did you consider for use in this project?

We looked at using cells and tissues grown in the lab to test treatments. The system we have isn't good enough to completely replace the use of animals. But using this system could give us information about the treatments effects that using animals cannot, because we can use different imaging systems. It can also give us an idea of if a treatment is likely

to work as hoped before any animals are used.

Why were they not suitable?

The cellular model can only show the effects of the treatment of a small part of the eye rather than the effect on retinal tissue supplied with a blood supply i.e. an animal. The cellular model is unable to show if there have been any effects on eyesight, which we can only show using animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals needed for each experiment will be based on our work from before and information reported about the assessments we are using. We can use this to estimate the numbers needed to show changes in the structure of the retina and in eyesight. This gives us the minimum number of animals needed to see a significant effect.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each experiment will be designed to get maximum amount of information from the minimum resources. We do long term experiments with the same animals using scanning methods that cause no pain. We are careful to give the animals breaks between scans, and have a limit to the number of scans we can do on each one to prevent stress build up. By doing these long-term experiments, we reduce how many animals are needed to see the developments over time.

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 house uses a breeding for use program, this means we only ask for the animals we need.

We have some problems with using the male mice for long term experiments, as they have bad reactions to anaesthetic. We will still use them for other assessments that don't need the animals to be put to sleep, such as vision assessment. They can also be used for tissue for molecular or imaging work.

As we are only interested in the eyes and sometimes the brain, we are happy to share other tissues with other researchers if they are usable.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 laser model for wet AMD is well established and produces a reliable and reproducible model. This means we don't need to use harmful genetic models. Since we know that it doesn't cause pain in people and it does not seem to cause distress in mice, it is a very useful technique.

The laser model of dry AMD we have been developing is also a reliable and reproducible model. Other models include genetic mutants or the use of sodium iodate. These methods can be harmful or invasive, but the laser model is a painless non-invasive model.

The procedures described for this project can be performed in humans with just eye drops and will be performed on mice under general anaesthetic.

Why can’t you use animals that are less sentient?

AMD is mainly an ageing disease, so an adult model is necessary to show the development over time. Long term experiments let us watch the development and produce more data from each animal. It is not technically possible to apply retinal laser to less sentient species e.g zebrafish or fruit flies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Basic best practice will be monitored by both the research team and the animal care staff to ensure animals are handled and cared for properly. Animals are handled with the tunnel method to reduce stress from experimental procedures. Animal weight is monitored at a minimum during every procedure.

Animals are particularly closely monitored for several days after laser burns, if they show signs of pain in that time they will be given pain relief. We will have an eye specific grimace scale for our animals, to improve monitoring.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the most recent guidance from the NC3Rs. We will also regularly check the literature for changes in best practice for the specific procedures and methods.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed of advances in the 3Rs using several online resources, such as the NC3Rs website, and resources from the European's Commission website. Relevant information will be passed on to animal workers and will be incorporated into protocols and

training as appropriate.

120. Behavioural ecology of wild birds

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

birds, behaviour, mating system, ecology, evolution

Animal types	Life stages
Blue Tit Parus caeruleus	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 objectives are to understand factors which lead to individual variation in reproductive success in birds. If we understand how individuals vary in their response to environmental stressors, such as pollution, such information can be used directly to inform population models which provide evidence used for conservation 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?

In a world with a changing environment, especially when those changes are driven by human impacts, it is important to understand the mechanisms that drive and limit adaptation and change within populations. This work looks at a fundamental aspect of the evolutionary process, reproductive success, and seeks to understand the interactions between individuals and their environments in predicting variation in reproductive success. By understanding more completely how animals select mating partners, and how those choices might be disrupted by man-made phenomena such as pollution and traffic noise, we will be in a more robust position to mitigate such effects if we need to intervene to drive conservation actions.

What outputs do you think you will see at the end of this project?

The outputs we predict from this work will be new information that will be published in peer reviewed academic papers.

Who or what will benefit from these outputs, and how?

These outputs will contribute to a body of knowledge on factors that influence reproductive success in wild animals. The immediate beneficiaries will be other academics working in the fields of evolutionary and behavioural ecology, but we expect the emergence of a greater understanding of environmental impacts that will ultimately inform efforts for conservation practices.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised through the sharing of data and collaboration with other researchers investigating similar questions, so that larger scale effects can be assessed.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using birds for this study because they provide an excellent opportunity to combine genetic, phenotypic and behavioural data from wild animals. They are readily observable in the field, and can be marked individually with rings to connect behaviour to other variables such as reproductive success and environmental variables. We will use nestlings and adults because we are interested in measuring reproductive success and genetic evidence has shown that the nestlings in the nest of a pair of birds may not necessarily be the genetic offspring of the mother or father attending them because females sometimes lay eggs in the nests of other females, and also mate with non-pair males when they are fertile.

Typically, what will be done to an animal used in your project?

Typically, an animal in this project will under go a mild procedure where a small blood sample is taken to provide tissue for DNA extraction or for hormone analysis. The animal will be captured from the wild for the purposes of ringing with a unique identification number, and following that, a blood sample will be taken. The bird is then released back into the wild after the process which would typically take 10- 20 minutes.

What are the expected impacts and/or adverse effects for the animals during your project?

The effects are likely to be minimal. Taking a small blood sample can provide a wealth of data, including parentage, sex, indicators of stress and genome information. The severity

is mild in relation to the potential gain in information, and the animals are released back into the wild at the end of the procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect any animals to suffer more than the mild procedure.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The research questions focus in the behaviour and ecology of the birds per se, rather than using them as a model organism to substitute another species.

Which non-animal alternatives did you consider for use in this project?

As a consequence, no non-animal replacement was considered.

Why were they not suitable?

Replacements are not possible for this type of study.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 an epidemiological study of individuals within a population, so inevitably relatively large numbers are used. We generally need to monitor all individuals within the study population to obtain data on the full spectrum of phenotypes and environments that they occupy.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This is an epidemiological study that is attempting to measure effects which are potentially quite small (e.g. typical effect sizes may be <20%), hence reduction in numbers sampled is difficult. Additionally, the unit of replication will be the brood or breeding pair, rather than the individual chick. As brood sizes can be up to 14 chicks, the number of individuals sampled will be quite large to achieve sample sizes suitable for powerful 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?

Experimental design will be based on strong statistical theory, in consultation with professional statisticians if necessary. Typical analyses would involve Generalised Linear Mixed Models, to control for random effects such as brood of origin.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 research questions focus in the behaviour and ecology of the birds per se, rather than using them as a model organism to substitute another. The bulk of the work uses bird species that are common or abundant, nest-box breeders, that can readily be handled and monitored without showing a negative response (e.g. by deserting their nests). The procedures are mild, and experience shows that the birds usually return to their normal behaviour within minutes following the procedure. No lasting ill effects were observed in terms of pain or damage, and no ill effects on reproductive success were found.

Why can’t you use animals that are less sentient?

Less sentient animals cannot be used because the research questions focus in the behaviour and ecology of the birds per se, rather than using them as a model organism to substitute another.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures are already well refined, but we always seek to improve our methods where possible. For example, when blood is taken for a source of DNA we can now take small samples due to advances in laboratory methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance on using animals in scientific research published by the Association for the Study of Animal Behaviour: https://doi.org/10.1016/j.anbehav.2019.11.002.

Also, the Home Office's advice note 02/2016, Working with animals taken from the wild.

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 regulations and best practice by reading updates and news published by the Home Office; we will also follow updates to the PREPARE guidelines https://norecopa.no/PREPARE for experimental design and ARRIVE guidelines https://arriveguidelines.org/ for reporting experimentation.

Home Office

121. Epithelial stem cell behaviour away from homeostasis; translational relevance.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Squamous epithelium, Development, Tissue injury, Aging, Cancer

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 this project is to investigate how the cells of the oesophagus (the gullet; the tube that connects the mouth to the stomach) and other related tissues (like the skin) adapt to perturbations such as aging, wounding and tumour 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?

Diseases of the oesophagus are very aggressive in nature, challenging to treat, and with poor overall outcomes. Oesophageal cancer, for example, represents the eighth most common cancer and the sixth most deadly worldwide, with a 10-25% five-year survival. Other oesophageal complications, such as injury, also have a poor survival rate of less than fifty percent. Hence, medical conditions of the oesophagus represent an important social and economic burden, with a significant proportion of our public health budget spent in treatments that are inefficient and, in most cases, unsuccessful. Thus, it is critical to

better understand this organ in order to identify clinical strategies that may help us improve the quality of life of patients suffering from these appalling diseases.

More specifically, in the proposed research we will use mice to investigate the way in which the cells of the oesophagus and other related tissues (such as the skin) function under normal conditions, and evaluate how their behaviour changes in response to one or a combination of tissue perturbations (situations driving the tissue away from normal health). These include:

- Aging
- Injury
- Early cancer formation

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 in our benefit and improve health. We ultimately aim to improve wound closure, minimizing scarring, and to treat cancer more effectively.
Additionally, by investigating cancer formation from its early stages, we expect to identify markers that can assist in the early detection of oesophageal cancer, 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 that line the oesophagus (epithelial cells) respond when not in normal health. Relevant findings will be compared with other related parts of the body (tissues), such as that of the skin, to identify whether our observations have a wider applicability, beyond the oesophagus.

In our publications, we aim to cover a number of relevant aspects:

Identify changes in the wound healing ability of cells during aging.

Define how changes in the immediate environment that surrounds a tumour affect cancer formation.

Reveal whether cells return to an early developmental stage when perturbed.

Uncover the impact of aging and wounding on tumour formation.

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 tissue to heal (tissue regeneration) and to develop cancer, 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 tumours. 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 organizations such as the Wellcome Trust and The Royal Society. This will mostly benefit the scientific community, particularly those with interests in tissue regeneration and cancer. In the long- term, our findings could be used to develop therapies to improve wound healing and treat oesophageal cancer. This will benefit patients suffering from cancer and/or injuries to the oesophagus and/or skin, 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 programme, the **management** of individual projects 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 formation of cancer in other organs such as the tongue and the cheek, among others.
- Moreover, in our effort to understand the behaviour of cells, we conduct the analysis of large-scale datasets. To maximize the outputs obtained from these complex datasets, we collaborate with experts in theoretical physics, mathematical modelling, and bioinformatics, 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 early cancer, 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: 38150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Traditionally, **mice** are used in our research field because they allow relatively easy genetic manipulation, something that facilitates

injecting substances that enable us **to track individual cells** over time and follow their behaviour,

manipulating cells **genetically** in order to understand the processes regulating their behaviour.

Our studies particularly focus on the **oesophagus** because of its structural simplicity. This facilitates the visualization and tracking of the cells we are interested in. Our results are then compared with other epithelial tissues, such as the skin, to identify whether our observations are relevant beyond the oesophagus.

This project will expand on our previous work by making use of all the methods that we have developed to study oesophageal/skin cell behaviour using animal models.

Mice will be studied at **different stages of their lifetimes** (from birth to aged stages) because we are interested in understanding why we exhibit a different wound healing response and cancer incidence as we age.
Typically, what will be done to an animal used in your project? Typical experiments
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 aging, wounding or tumour formation). To address our research questions, animals will typically undergo one of three main types of experiments:

Aging: Animals will be administered various substances to label their cells, by injecting them into their abdomen or under their skin. 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 aging on cells.

Tumour formation: Animals will be treated with low doses of carcinogens (cancer-causing agents), typically via drinking water supplemented with sweeteners (such as Ribena) to alleviate any bitter taste. Animals may also be injected with various substances to label their cells (as above). Animals will then be allowed to grow old and tumours to form. 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 labelled cells will be analysed to

investigate how tumours emerge. Additionally, blood samples may be collected in order to identify markers of potential use to improve cancer detection in the clinic.

Wound healing: Animals will have a surgical procedure. Surgery will be completed under general anaesthesia so that animals remain in a state of sleep/unconsciousness throughout the procedure. During this procedure we will wound the mouse skin (up to two wounds of no more than 1 centimetre in size, separated by at least 1 centimetre). Wounds may or may not receive a graft (a piece of living tissue that is transplanted surgically). Grafted and non-grafted wounds will help us understand different regenerative/healing processes. A small number of animals may be treated with various substances to label the cells and track their regenerative response. Subsequently, samples will be collected after humanely killing mice at different ages from birth and up to 15 months of age. These experiments will shed light on how wound healing 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. We anticipate that our work will have a minimal impact on the health and welfare of the animals, and will not lead to long-lasting pain, suffering, or distress. The genetically modified mice used are not expected to show deviations from normal health.

Substances administered during the course of this project are not expected to cause any harms that affect the animal's day-to-day life. In certain instances, treated animals may experience transient episodes of weight loss up to 15%, from which they usually recover after 2-7 days.

Carcinogens (cancer-causing agents) used in this project (such as DEN, derived from tobacco) have not been observed to affect the normal behaviour of treated animals. However, as animals bearing tumours age, they may sporadically present transient weight loss of up to 15%. In very rare instances, these mice become sick in which case they are humanely killed.

Wounding/grafting surgeries to the skin. These will be limited to wounds of up to 1 centimetre in diameter, in order to ensure that the injury does not cause distress to the animal. All surgeries are carried out under anaesthesia and animals receive pain relief medication that will remove any discomfort during the procedure and in the recovery period. Mice normally recover from surgeries within 10 - 15 minutes and show normal behaviour. Sporadically mice may display a small amount of swelling in the wounded/grafted area. This naturally disappears 7 days after the procedure. This swelling has not been observed to cause distress to the animals.

When **baby mice** (mice that have not been weaned) undergo a procedure (typical duration of 5- 20 minutes), they are returned with their brothers and sisters to their mother immediately afterwards. The mother has always been observed to welcome her babies back.

Expected severity categories and the 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 will use **mice**. 38% of the protocols have been categorised as mild, and 62% of the protocols are classified as moderate, due to their nature (including carcinogen treatment and surgical protocols). However, from our previous experience, we foresee that the **actual severity** of our mice will exceed:

-Mild: 90% (approx.)

-Moderate: 10% (approx.)

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal experimentation is required to obtain relevant information on cellular behaviour within the living organism. In order to identify treatments of relevance to improve wound healing and reduce tumour growth, we need to understand the way in which different cell types interact with each other. The oesophagus and skin are complex organs formed by different layers and tissue compartments that contain different types of cells. In response to an injury or genetic alterations leading to cancer, all these cells need to communicate in order to elicit a coordinated response that results in the healing of the wound or the formation of a tumour, 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?

In my laboratory, we have developed methods to grow tissues outside the animal. We can currently grow epithelial tissues/cells (e.g. from the skin/oesophagus), as well as underlying supporting tissues (called stromal cells). This approach represents an outstanding tool to replace the use of animals for short-term experiments studying wound healing and tumour formation. At the moment, all our projects make use of this technique, wherever possible.

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 (such as epithelial, mesenchymal, and immune cells) interact with each other and contribute to aging, wound healing and tumour formation 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 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 minimize 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 on 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.

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 tongue and the cheek. 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 oesophagus can produce sufficient cells to perform 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.

Optimization of skin transplantation technique (grafting): We have implemented diverse adjustments in skin transplantation experiments, which increase the quality and statistical value of our data while substantially reducing the number of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

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 the cells allowing us to track their behaviour in the tissue. Hence, the genetic alterations we propose are not expected to lead to any adverse effects.

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 drugs to understand how cells work and explore the earliest stages of **tumour formation** (carcinogens). Given our interest in early cancer, these treatments use low doses of these drugs. Our experiments have been optimized for over a decade, which allow most mice to live without complications for over a year. Carcinogen administration is typically performed non- invasively via drinking water.
- For **injury and tissue transplantation** (grafting) experiments. These methods have been significantly refined in my laboratory over the last five years. For skin wounding, we use instruments known as “punch” biopsies or biopsy forceps, which are specially designed to collect tissue specimens in the clinic. These instruments allow us to perform small skin wounds that favour rapid healing and diminish animal discomfort. Over the years, we have also optimised the size of the grafts, reducing it when compared to standard practice. We currently perform grafts of a maximum of one centimetre in diameter with optimal results. The most significant improvement of our refined method resides in the bandaging used to protect the wounded area. For this, we use transparent Tegaderm medical dressing, which constitutes a very thin transpirable barrier that protects the wound and keeps it aseptic, while allowing animals to move freely. This significantly reduces the stress experienced by the mice while recovering from surgery. When compared to classical bandaging, Tegaderm has the added advantage of allowing constant monitoring of the wounded area after surgery. Animals typically remove the Tegaderm by themselves on day 4-5 after surgery; by then, the exposed wound/graft allows for closer monitoring and mice are completely freed from any dressing.

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 cancer. Given the differences observed in wound repair and cancer incidence 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:
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.
- 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.
- Wounding of the skin is performed using tools called "punch biopsies", instead of scissors. This allows to produce clean skin biopsies, which favours rapid healing and diminishes animal discomfort. The size of the grafts has been optimised to increase grafting success. The refinement of this technique also allows us to reduce the number of animals used.
- Skin wounds will be on body sites excluding face and paws to minimise discomfort.
- We have refined the skin wounding technique to use minimal bandaging/dressing, in order to avoid any restriction of animal movement while protecting the implant until grafted.

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 ameliorate 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 subcutaneous [injection under the skin]) 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. Animals have enrichment in their cages (such as wood sticks, bedding and nesting material) for extra comfort and enhanced mental and physical health.

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 veterinarian 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

122. Molecular mechanisms in cardiometabolic disease: breeding and maintenance of genetically altered animals

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - (ii) 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

Breeding, Rodents, Cardiovascular disease, Diabetes, Heart failure

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To maintain breeding colonies to produce genetically altered mice for projects involving cardiovascular and metabolic scientific 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?

Animal models remain indispensable tools to investigate the molecular causes of human disease and to identify new targets for prevention, diagnosis and treatment. The combined effects of cardiovascular disease and diabetes on human health (cardiometabolic disease) are of major concern currently due to increasing number of people with obesity or diabetes

and more people likely to develop these diseases as they age. The reasons by which obesity or diabetes increase the risk of cardiovascular diseases are complex and incompletely understood. Genetically altered mice allow us to study the effects of individual genes in the molecular pathways which contribute to diabetes and cardiovascular disease.
Carrying out this work will allow us to identify new ways of preventing and treating diabetes and cardiovascular diseases.

What outputs do you think you will see at the end of this project?

1. Provision of genetically altered animals for scientific research projects investigating the role of specific genes in cardiovascular and/or metabolic disease.

2. Maintenance of rodent colonies in high-health status for use in scientific research projects

Who or what will benefit from these outputs, and how?

The outputs will benefit scientific researchers undertaking biomedical research. Mice bred on this licence will allow researchers to study the effects of specific genes on the development of obesity, diabetes, heart and circulatory disorders. Ultimately the research enabled by these outputs may lead to the discovery of new ways of preventing, diagnosing, monitoring and treating disease in humans.

How will you look to maximise the outputs of this work?

Mice bred under this licence will be used in experimental licences held by the applicant or collaborators covering basic and translational research into cardiometabolic disease. We strive to present our research findings at national and international scientific conferences and publish our work in high impact scientific journals. We welcome requests for collaboration from the wider scientific community. We engage in local, national and international research groupings and consortia to share knowledge and ideas.

Species and numbers of animals expected to be used

- Mice: 18 000 animals over five years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use genetically altered mice in scientific research because it is relatively easy to alter the DNA in mice to study the effects of particular genes. Use of a mammal enables experimental results to be relevant to humans. There is a wide range of genetically altered animals available to the research community; new genetic alterations can be created by scientific or commercial organisations as new data become available. Once a genetically altered line is created, mice are bred locally to provide animals to researchers at that institution. In many cases, mice from different genetically altered lines are bred together to form colonies with multiple genetic alterations. Breeding involves mating of adult animals.

Pups are maintained at life stages from neonate to adult to provide animals for scientific research projects authorised by other licences.

Typically, what will be done to an animal used in your project?

Male and female mice will be paired and allowed to breed to generate genetically altered pups. In most cases breeding also generates non-genetically altered pups which are used as controls. The presence of altered genes in pups will be determined by analysing a small piece of ear or tail. Animals will be maintained using standard husbandry procedures until they are transferred to other projects or used to replace breeding stock. Animals will be periodically visually inspected and weighed. Animals which are not used for other projects or breeding will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Sampling of ear or tail may cause transient pain to the animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The work will be of sub-threshold or mild severity limit with no suffering which is more that mild or transient.

The genetic alterations we plan to study are not known to cause harm or suffering to animals. However, when new genetically altered lines are bred or existing strains are crossed together we cannot entirely rule out the possibility of pups exhibiting clinical signs. If any new line demonstrates a phenotype which is more than mild and which develops before the animals are transferred to an experimental licence, breeding will be suspended and this will be discussed with the Home Office Inspector.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project is intended to generate genetically-altered rodents for use in other scientific research projects. Although biomedical research can often be carried out in isolated cells or tissues, in some cases the complex interactions between biological systems and the circulation can only be studied in a living animal. For example, the influence of genes on diabetes and its effects on the heart and circulation can only be studied in the intact animal. This is because diabetes affects many circulating substances including insulin,

glucose and fats which all influence the heart and blood vessels. Specific rationale for the use of animals generated by this project in scientific research is provided by the licences giving authority for that experimental work.

Which non-animal alternatives did you consider for use in this project?

We conduct many aspects of our research without the use of animals these include research in silico, in cultured cells and in tissues obtained from humans undergoing surgery.

Why were they not suitable?

The complex nature of cardio-metabolic disease involves interactions between multiple circulating and locally produced factors which cannot adequately be modelled in vitro. There are no suitable in vitro models for many of the cardiovascular pathologies which affect humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals based on the number of experimental animals required by the licences to which this project will supply animals, taking into account breeding strategies and requirements for controls. This licence will also provide tissues for scientific research from genetically altered mice killed under Schedule 1.

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 generated in this breeding licence is reviewed regularly and aligned with the number of animals required by the project licences it supplies. Experimental design utilises tools such as the NC3Rs experimental design assistant and is described in the licences for which this licence provides 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 design our breeding colonies and schedules to maximise generation of animals and reduce unnecessary production. This requires careful selection of which types of genetically altered animals to breed together to generate animals required for the research projects this project supplies. In a small number of cases, particularly when it is necessary to alter several genes at the same time, the required genetic alterations only occur together in a small number of offspring. In most cases, however, we can plan breeding so that all pups can be used as experimental animals or controls. We use an efficient commercial service to detect the genetic alteration in pups so that we can make early decisions on the use of animals. We use a digital animal management system to allow us to review breeding colonies on a weekly basis and hold monthly meetings with our

research group to ensure the most efficient use of 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.

This project provides for the breeding and maintenance of genetically altered mice for use in other projects. Animals will be transferred to licences for use in other projects, used to replenish breeding stock or will be humanely killed. Genetic alterations are of genes implicated in the molecular pathways which lead to the development of diabetes or cardiovascular disease.

Why can't you use animals that are less sentient?

The aims of this project can only be achieved by using mice at the ages required for breeding or for supply to other projects. Mice are the lowest order mammals in which which is feasible to readily alter genes. Although some aspects of molecular cardiovascular research can be carried out in zebrafish, it is not possible to model more complex human disorders such as diabetes, atherosclerosis or cardiac failure in zebrafish.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use contemporary breeding and husbandry methods and keep ourselves updated on advances in laboratory animal husbandry and welfare. We strive to provide an environment conducive to successful breeding - for example by keeping breeding colonies in dedicated rooms and limiting access to non- essential personnel.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our experimental work will follow ARRIVE and PREPARE guidance. This is covered separately in the licences providing authority for experimental work.

We will follow the NC3Rs resource for breeding and colony management written by a working group in response to returning to scientific research after the COVID-19 lockdown (https://www.nc3rs.org.uk/breeding-and-colony-management).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our group stays informed through the NC3Rs website and relevant information, including the NC3Rs newsletter, is circulated within our institution by email to all personal and project licence holders. We attend local events organised by our Animal Welfare and

Ethical Review Committee and information sessions on NC3Rs funding streams organised by our institution's Research & Innovation Service (last held 10th November 2020). We share best practice within our institution and have well developed interdisciplinary to facilitate this. We hold regular local user-group meetings for project licence holders at which the group receives updates on any changes to best practice or requirements.

123. Development and function of the neonatal immune system

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

neonatal, immune cells, development, leukaemia

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 shall look at the development of cells that make up the immune system and how they work to see if this differs as animals age. We are particularly interested in how an animal may respond to particular infections and disease that cause mortality and morbidity in infants and children but often not 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?

Many babies (both those born early and at term) die from infections and problems that have little adverse effects in adults. Furthermore, those that survive infection may be left with neurodevelopmental abnormalities and life-changing disabilities, such as cerebral palsy.

It appears that children may protect themselves from such challenges (this protective role is a main function of the immune system) in a different way to adults using different cells

and different mediators. Understanding how infants develop their immune system and respond to these different challenges may help us better protect infants during these vulnerable early life stages.

What outputs do you think you will see at the end of this project?

This proposal can provide data and key insights that would improve our understanding of how immune cells develop in the neonate and potentially change throughout the lifespan of the individual.
Furthermore, the way in which infants respond to challenge (both from infectious agents and microbial colonisation) will guide the development of potential therapies to enhance protection in the young. This project will lead to enhanced knowledge disseminated through publications and conference attendance.

Who or what will benefit from these outputs, and how?

By uploading data promptly and making it accessible as soon as possible, we hope to enhance the impact and benefit, at least for academics for whom these data are interesting in the short term.

Although this research project on its own does not provide a direct road to any therapy, a further understanding of the role of the immune system may result in further studies and potentially clinical trials in this vulnerable group. Greater understanding of the development of the immune system by this proposal will provide a basis to understand how infants respond to infection and how we can better protect infants from infection, a major cause of death in the very young. Identification of true infection in human neonates is very challenging and if we can identify immune changes that are specific to infection, this will also reduce the overuse of unnecessary antibiotics and hence help to reduce antibiotic resistance.

How will you look to maximise the outputs of this work?

The usual routes of dissemination of research data will be undertaken in the form of transmissive presentations at conferences, invited lectures and publication in peer-review journals. By attending conferences, we hope to enhance the outreach of the data to all for whom it could be interesting. We will endeavour to publish all of our findings, in particular those that were unsuccessful, in a bid to prevent others taking the same path.

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.

The mouse is the long-stranding choice of immunologists and has successfully identified the key operating principles of the immune system on which major clinical advances are based.

Our whole area of research is based upon understanding how infants respond differently to challenges than adults. Hence, we will use mice of different ages (from a few days old to adulthood) to study these questions. As such challenge experiments cannot be performed in human children, the mouse is the most functionally relevant model available. We will use around 3000 mice in total during the period of this licence (5 years)

Typically, what will be done to an animal used in your project?

Many animals will be used solely to provide different cells and tissues from mice of different ages after culling for study in the laboratory and hence will experience minimal harm.

Some mice will be given very mild doses of infectious agents or other agents (eg milk formula) by the most suitable route of administration leading to minor/moderate effects on the animals who will be killed not long (usually within days to weeks) after the procedure begins.

Some mice will be given human cells derived from healthy or diseased individuals and may experience some ill effects but these will be monitored and no mouse will experience any severe effects as they will be culled before reaching this point.

What are the expected impacts and/or adverse effects for the animals during your project?

By far the majority of the mice on the breeding protocol will experience no ill effects and mice used to provide tissues will similarly experience mild severity.

The use of challenge models may have different potential outcomes depending on the age and the immune status of the host. Many assessments in very young mice rely on weight loss (more correctly termed weight retardation although this retardation only lasts temporarily after which the mice would develop at the same rate as normal). Some other potential signs of distress may include hunched posture, lack of appetite and minor weight loss. Nevertheless, challenged animals will be monitored several times a day during periods of potential disease to ensure that should they develop any serious clinical signs, appropriate action is taken. Much of the work is aimed to identify early clinical signs of infection and as thus, mice will be culled very early post challenge and prior to the development of any symptoms.

When leukaemic cells are used for adoptive transfer, the animals may develop leukaemias, phenotypically similar to the human disease. The time to develop leukaemia will vary depending on the primary leukaemia engrafted but usually after about 7-10 weeks. The mice are not overtly affected by the leukaemia growth. It is expected that the mice will have a mild systemic effect.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of the mice (the only animal utilised) on this licence (2/3rds) are used in the breeding and maintenance protocol to provide mice and tissue post culling and hence will

experience no ill effects (so called sub threshold) whatsoever.

The remaining mice in the other protocols may receive invasive procedures. Although some of this will still be mild (30% of these mice) more moderate severities will be experienced by around 70% of mice in these protocols (totally around 600 mice in total over the 5 year licence)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 way to monitor the different cells and mediators made in normal human children at different ages as these babies are not in hospital and so no blood can be taken. Similarly, it is not ethical to take excessive blood from infants unwell in hospital. To follow immune cell development over time, therefore, we must take samples from mice at different ages. Similarly, leukaemia samples taken from people will not usually grow in the laboratory but only in an animal host. The core of our studies is to follow and dissect the molecular basis of immune cell development in neonates and response to challenge. It is impossible to obtain longitudinal samples from healthy term human babies and as such following immune cell development in mice is the only option.

Which non-animal alternatives did you consider for use in this project?

Studying development within the thymus itself is achievable using well established in vitro culture systems (such as fetal thymic organ culture) and these will be utilised wherever possible.

Organoids (such as gut organoids) can be used to look at gut immune cells and will also be done in isolation but cannot replicate the systemic effects of the immune system even after challenge in one site.

Why were they not suitable?

Mice are the best model to use as these can be easily modified and by far the majority of reagents are designed for use in this system meaning more useful data can be obtained from every mouse.

Individual mice can also be followed over time to reduce the number of animals used in total.

Whilst organ cultures can be useful (and we will use them to look at individual cell features), it is the interaction between different organ systems that control the response to challenge. This cannot be studied in single organoid 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?

Having held project licences (as primary holder or deputy) for over 20 years, I am well aware of how many mice are needed to maintain mouse colonies in general.

Where pathophysiologic responses are to be assessed, we use the minimum number that will provide enough high quality data to answer the question. These assessments are based on our own previous work and also other's peoples work where they have used techniques that we have not used in these contexts.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In many cases, the numbers of animals required will be reduced by longitudinal measurement of responses, by serial blood analysis or by optimised protocols for intravital imaging. Hence, T cell development may be measured weekly in a set of six mice over a period of six weeks, rather than requiring six mice to be sacrificed weekly across that period. Such longitudinal usage provides essential information on the development of the immunological landscape. This may not be possible if sampling from very newborn mice but this will not be necessary in all cases as 7 day old mice are more akin immunologically to a newborn human.

Unnecessary variation in the animal cohort will be minimised by use of gender and age-matched controls housed under identical conditions. As part of good laboratory practice experimental protocols for each experiment defining the objectives, experimental procedures and intended effects, and endpoints will be circulated to all those involved in the care of the mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As well as good design, careful optimisation of reagents will lead to a reduction in animal use eg : ready availability of reagents in defined forms that permit high reproducibility of experiments while using only small numbers of mice; our own previous experience of the infection challenges and xenograft models and/or ready availability of protocols within the community ; reagents exist that permit the most thorough analysis of response, and hence the maximum amount of information to be obtained from single 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.

Much of the work is sampling from mice without any challenge to look at the normal development of the immune system. This is in wild type mice and also in GA (NSG that are immunodeficient) mice that have been given human cells so as to make an equivalent human immune system. Both of these methods will cause minimal distress. Most animals produced under the breeding protocols are not expected to exhibit any adverse phenotypes and most immunodeficient mice are also unlikely to show adverse symptoms because of the high health status of the animal facilities. Otherwise, we will use mouse models of inflammation (limited to intestinal inflammation) and infection and one tumour model, the latter rarely shows any ill-effects as mice are culled as soon as the leukaemia is readily detected in the blood.

All mouse models used will be assessed such that the minimum severity is reached to show the required effects. Mice will be killed at stages of disease progression consistent with those widely used throughout UK and international scientific institutions. Animals exhibiting any unexpected harmful phenotypes will be killed by schedule 1, or in the case of particular scientific interest, advice will be sought from the local Home Office Inspector.

Invasive mouse procedures required are very minimal. Analgesics will be used as required.

Why can’t you use animals that are less sentient?

The mouse is the ideal organism for our investigations.

It is the long-standing choice of immunologists and there is an immense spectrum of reagents available permitting the thorough, incisive, and comprehensive obtainment of information from the experiments undertaken. The NSG mouse (the only GA strain being used) is ideal to transfer human cells (both wild type and leukaemic) and the high health status of the animal facilities means these animals are generally very well despite being immunosuppressed. Non invasive methods of longitudinal monitoring, e.g. by intravital microscopy, reduce the numbers of animals required for parallel, staged analysis of phenotypes. The questions posed by this license necessitates the use of young animals.

Using less sentient animals presents difficulties in delineating the functions and relationships of different immune cells as well as obtaining data that can be translated to the clinic.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where necessary, we will refine the procedures we are using by increased monitoring e.g. FELASA “Working Group on Pain and distress”, EC “Endorsed Severity Assessment” and NCRI guidelines will be followed and used to determine the earliest endpoint possible on an experiment by experiment basis to allow a valid scientific outcome. Nevertheless, this will only be relevant in a few cases as the majority of our animals will experience mild

adverse effects

Assessment will be made of any pain and distress, as measured by normal and provoked behaviour; movement; physical signs such as altered respiration rate; animal posture (huddling or hunching) skin and coat changes such as piloerection or overgrooming; inappetance or inactivity; body weight, food and water consumption; inflammation of injection sites; and comments on the animal's general appearance.

All mouse models used will be assessed such that the minimum severity required to identify effects will be employed. We will be careful with our choice of reagents and utilise doses known to give the least adverse effect whilst still achieving the aims. Mice will be killed at stages of disease progression consistent with those widely used throughout UK and international scientific institutions

Invasive mouse procedures required in this application are minimal. Appropriate analgesics for each procedure will be used as required

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As mentioned above, where needed, we will follow best practice guidance published by:

FELASA "Working Group on Pain and distress"

EC "Endorsed Severity Assessment"

NCRI guidelines

National Centre for the Replacement, Refinement and Reduction of Animals in Research guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep updated with the guidelines specified in the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). and ensure that all my staff are aware of any changes to the guidelines and ensure we try, at every juncture to implement these advances effectively.

124. Inter-connected studies of Alzheimer's Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Alzheimer’s disease, Transgenic mice, Prion-like strains, Amyloidosis, Behavioural studies

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, aged, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to breed and maintain genetically modified animals that will be used to study the basic biology of prion-like proteins in order to understand the role of abnormal protein aggregation in neurodegenerative diseases, further understand strains and pathophysiology of Alzheimer’s disease (AD) and to develop diagnostic techniques, understand the kinetics and spread of prion-like assemblies, characterise the infectious and neurotoxic species of AD and other neurodegenerative diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The purpose of this work is to embark on basic research likely to yield results that can be translated into more reliable AD disease diagnosis and ultimately treatment in patients.

What outputs do you think you will see at the end of this project?

Prion diseases are an important model for several other brain neurodegenerative disorders, including Alzheimer's disease, because it appears likely that very similar processes may be involved. Currently, there are no treatments or cure for Alzheimer's disease and for many of the so-called prion-like diseases. Confirming and understanding the aggregation mechanisms for the different misfolded proteins is paramount in order to develop therapies for the neurodegenerative diseases.

Who or what will benefit from these outputs, and how?

This research is critical to understanding the mechanisms of diseases associated with protein aggregation such as AD which will guide the development of better early disease diagnosis and treatment. Ultimately, patients will benefit from the outputs of this research.

How will you look to maximise the outputs of this work?

Our research is strategically inter-connected, thus maximising a range of skills and expertise to ensure that the most important questions in AD biology are being tackled from many different angles. In addition to the internal close working relationships, we also work with several external collaborators on specific aspects of our research programmes. The Institute provides an internationally recognised centre of expertise in research in neurodegenerative disease, and provide specialist technical and advisory expertise to academic centres, industry, WHO and Government at a national, EU and international level. Any new knowledge acquired through the conduct of this project will be published in the literature and we will facilitate the dissemination of knowledge and experience nationally and more widely.

Species and numbers of animals expected to be used

- Mice: 54,700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used as their genetics, physiology and neuroanatomy are very well-characterised and their breeding time and lifespan allow experiments to be performed on a manageable and affordable timescale. Their similarity to humans in terms of genetics and neuroanatomy, and their genetic tractability, which allows for the generation of genetic models of human diseases, has led to the generation of many successful models of neurodegenerative disease and the identification of potential therapeutic avenues. For these reasons, the mouse is our model of choice for gaining new insight into neurodegenerative disease mechanisms that are likely to be relevant to humans. Different stages of the mouse will be used as appropriate for each protocol.

Typically, what will be done to an animal used in your project?

This project has 9 Protocols in total, and procedures will vary depending on the specifics of the protocol. Animals produced under Protocol 1 (Breeding and maintenance of GA

animals; Mild threshold) will not undergo procedures that involve more than the mild severity threshold. Mice that are expected to develop a harmful phenotype (e.g.TauP301S mice) will be bred and maintained under Protocol 2 (Breeding and maintenance of GA animals; Moderate threshold) in which specific guidelines on how to monitor, care for and alleviate adverse effects will be followed. Some animals will be infected with amyloid-β or other prion-like proteins, human growth hormone or neurotoxic substances under general anaesthesia (Protocols 5, 7-9). A subset of mice will be used for behavioural studies (Protocol 6), and collection of brain tissue for neuronal cultures or in vitro assays (Protocols 3 and 4).

What are the expected impacts and/or adverse effects for the animals during your project?

Animals undergoing surgical procedures carried out aseptically under general anaesthesia may rarely develop post-operative complications. Such animals will be humanly killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery.

Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Any animals that fail to do so or exhibit signs of pain, distress or of significant ill-health will be humanly 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) will be humanly killed by a Schedule 1 method.

In all other cases, any animal showing any deviation from normal health or wellbeing will be immediately humanly killed by a Schedule 1 method, unless the animal is under observation for neurological dysfunction according to either the “Criteria in AD mouse models” or the “Endpoint Monitoring for hTau P301S mice” detailed in this licence below.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of mice (84%) are expected to have severity sub-threshold, 8% mild and about 6% used in transmission experiments will have moderate threshold. It is anticipated that the severe threshold may be reached in up to 2% of the animals used, but animals will not be deliberately allowed to progress to this stage.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 fundamentals of prion-like diseases such as AD and improve decontamination methods and diagnostic tools, effective and appropriate experimental models are essential. We have cell culture facilities for studying some of the cellular mechanisms seen in AD but they are not specific, and need to be further developed. When fully developed and refined, the use of these new cell lines will allow us to perform some of our prion-like infectivity studies in vitro. However, until this test tube work has been successful, key prion-like disease parameters such as behavioural changes, brain damage and the spread of the abnormal protein within the body leading to fatal brain damage, can only be studied in an animal.

Which non-animal alternatives did you consider for use in this project?

Cell culture and aggregation cell assay developed in-house will be used for some of our studies. We have used and plan to continue using the clinical and post-mortem data of different cohorts of patients to study the strain subtypes and characteristics of AD. However, the use of the whole animal is unavoidable for the study of some key prion-like disease parameters such as behavioural changes, patterns of brain damage and the spread of the abnormal protein within the body leading to fatal brain damage.

Why were they not suitable?

Cell culture facilities for studying prion-like proteins in the test tube are suitable to an extent, but not every biological parameter can be studied in culture; some parameters can only be studied in the whole animal. Human brain material is a good source of bonafide prion-like proteins but it needs to be bioassayed in a whole animal and characterised to help us to understand the cause of the aggregation and misfolding.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

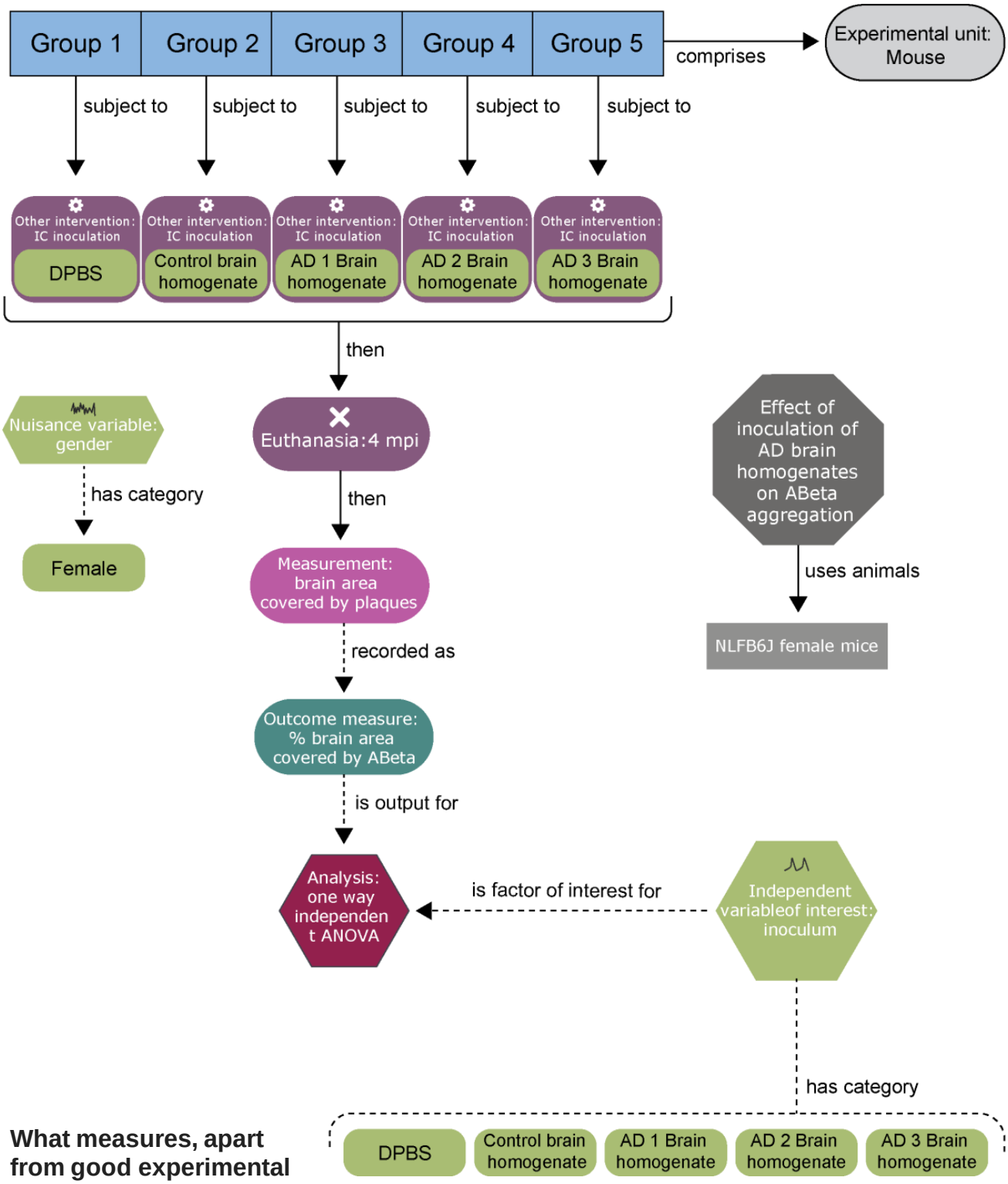
For comparison of protein misfolding aggregation times and strain-specific patterns of neuropathology, groups of mice of approximately 10-20 will be used based on what is known about how particular prion protein-like inocula behave in specific mouse strains. Large scale amplification of prion-like proteins such as Aβ or tau for biochemical studies involves larger numbers of mice (up to 100) that are required in order to isolate purified assemblies in the microgram quantities required for structural analyses. For transmissions using novel mouse or human prion-like strains, the transmission properties are unknown and the incubation time may exceed the life-expectancy of the mouse. In these instances, experimental output is often qualitative therefore group sizes of 10-20 will be used as this allows for sufficient tissue to be available at the end of the experiment.

For all our research we will ensure that the smallest number of animals will be used consistent with achieving a clear experimental result. We also regularly check our mouse

colonies to ensure efficient colony management.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We plan to reduce the number of animals used, by setting up a representative control group in the first experiment. Subsequent experiments of the same nature will not include new controls every time we perform a similar experiment, but instead reference will be made to the initial control data. The NC3Rs' Experimental Design Assistant (EDA) will be used in the design phase of individual experiments where short incubation periods are expected. The graphical output of a typical experiment designed using EDA is shown below. The full EDA report is too long to present here, and will be made available if requested.



What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will ensure that animals are bred to requirements, and that all animals left on the shelf have a justifiable reason for being kept. We are working to develop new infectivity and toxicity assays using cell lines and IPSCs, so even fewer transgenic mice will be required for routine bioassays.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 breeding all processes will be constantly monitored and any potential inefficiency likely to lead to excessive use of animals will be dealt with without delay.

If any deleterious phenotypes emerge from homozygous lines, such lines will be maintained as heterozygous lines.

Protocols 5-8 which would ordinarily be mild are assigned moderate severity limits because some of the mice in these other protocols could be inoculated with neurotoxic substances under general anaesthesia. It is anticipated that the severe threshold may be reached in up to 2% of the animals used, but animals will not be deliberately allowed to progress to this stage. By using anaesthesia and pain killers as necessary, and through careful monitoring, animal suffering will be reduced to an absolute minimum. We will minimise harm by implementing high standards of care for each animal, by ensuring that the maximum severity for all protocols is either mild or moderate and by using well-defined humane end-points

We are in the process of acquiring compact behavioural equipment called IntelliCages which we plan to use that are designed to allow mice to be studied in their home cage for the duration of the observation period for any behavioural and/or memory deficits, without human intervention. The resulting reduction in the release of stress hormones ensures that reliable data can be collected automatically from a smaller number of mice, and also ensure the repeatability of the data.

Why can’t you use animals that are less sentient?

Whenever possible we do use cell culture assays for our studies, but there are still some aspects of neurodegenerative disease research that are only possible in animal models. For instance, we are interested in the deposition of amyloid in the brain blood vessels, but this cannot be readily studied in a less sentient model such as Drosophila (fruit fly).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

By using anaesthesia and pain killers as necessary, and through careful monitoring, animal suffering will be reduced to an absolute minimum. We will also minimise harm by implementing high standards of care for each animal, by ensuring that the maximum severity for all protocols is either mild or moderate and by using well-defined humane end-points. The acquisition of a new behavioural equipment that allows the behaviour of mice

to be studied in their home cage and in social groupings for the duration of the experiment represents a significant refinement. The IntelliCage used together with a detachable Social Box, will allow the following behavioural parameters be measured automatically without operator intervention: territoriality, bonding, dominance, social structure, spatial preference/avoidance and novel object recognition. This minimal handling will result in less stress in the animals and is likely to result in more reliable data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to follow the NC3Rs' ARRIVE Guidelines 2.0, and LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.ubs.admin.cam.ac.uk/files/lasa_aseptic_surg.pdf) as best practice to ensure that our experiments are conducted in the most refined way. In addition, we will adhere to the Standard Dosing Information inserted in this PPL.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

In keeping with the Unit's commitment to the 3Rs promotion, a 3Rs Champion has been appointed to lead our efforts to comply with all aspects of the 3Rs. The 3Rs are a regular feature of the meetings of the AWERB and local Animal Research Scientific Committee (ARSC) which both monitor and ensure that animals are bred to requirements, and that all animals left on the shelf have a justifiable reason for being kept. The ARSC meets once a month and the 3Rs Champion, who monitors information coming out of the NC3Rs and other relevant sources, has the opportunity to report on 3Rs advances that can be implemented in our practices during the duration of the project.

125. Breeding and maintenance of germ free and gnotobiotic animals service licence

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Breeding, Microbiome

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 this project is to act as a service licence to breed, maintain and supply mice for microbiome research. Microbiome research investigates the impact of gut bacteria in 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?

This work will ensure a steady supply of germ-free mice and mice with known germs for the use on other experimental licences. These mice will be used to understand the role of

the gut bacteria (mouse microbiome) in disease and how it can be changed for therapeutic effect. This could include identifying mixtures of beneficial, health-associated bacteria which have the potential to be used to treat diseases such as cancer and digestive disorders in clinical trials.

What outputs do you think you will see at the end of this project?

The outputs from this project are:

production of demand matched animals for research thereby reducing animal wastage,

the production of animals with known gut bacteria required to answer specific research questions and

the communication of information arising from the breeding and maintenance of these animals to other researchers so that they might benefits from the knowledge.

Who or what will benefit from these outputs, and how?

In the short-term, mice produced on this project will enable microbiome experimental studies to be carried out by researchers on other projects.

In the long-term this research may lead to the development of microbiome therapies to benefit patients with diseases such as types of cancer including melanoma and lung cancer and intestinal disorders such as ulcerative colitis and crohn's diesease.

How will you look to maximise the outputs of this work?

Knowledge generated from the breeding and maintenance of these animals will be communicated with collaborators and other researchers working on similar projects.

Species and numbers of animals expected to be used

- Mice: 2500 germ-free and 1500 gnotobiotic

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 ideal for studying the microbiome. The mice we will breed and maintain are wild type (containing no genetic alterations) however they contain no microorganisms such as bacteria, fungi and viruses.

The project breeds of mice at all life stages, excluding aged mice, as mating and weaning are required to establish mouse colonies.

Mice share the same types of bacteria with humans and are small and easily housed in an extra clean environment in the animal house. The housing we use is called an isolator and everything that enters is sterile (e.g. the water, air, bedding etc.) to create a germ-free

bubble.

Typically, what will be done to an animal used in your project?

To maintain a germ-free colony it is necessary to breed mice within germ-free bubbles called isolators.

Mice will be mated in pairs (one male and one female) or trios (two females and one male) and productivity monitored with germ-free mice typically mated up to an age limit of 12 weeks. Offspring are maintained to 5-8 weeks of age before being transferred to other projects that require the use of these animals.

In some cases, germ-free mice are given specific gut bacteria from other mammals, to produce gnotobiotic mice with a known gut bacteria. The gut bacteria is administered via the mouth into the stomach through a tube.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding of animals is not expected to produce any adverse effects. For natural breeding issues, such as littering problems, advice will be sought from a Named Animal Care and Welfare Officer or the Named Veterinary Surgeon.

Germ-free mice can develop abnormalities within the gut such as an enlarged caecum (pouch connecting the small and large intestine) as they age. In turn, the size of the caecum may trigger a torsion of the intestine (twisted caecum). This causes chronic diarrheal status and death of the mouse in up to 3% of the animals. The age of breeders, number of litters and breeding performance will be monitored to minimise the impact of the enlarged caecum with age.

Mice will be checked at least twice per day and those observed to be less active, showing poor coat condition, abdominal distension, or other clinical signs that in any way compromises normal behaviour will be humanely killed.

Very rarely, gastrointestinal tract damage may occur during administration of microbiota by the mouth. This allows toxic material from the live bacteria to be released into the bloodstream causing infection and possible death within approximately four days. Animals displaying signs of infection, such as acute piloerection and lack of responsiveness when provoked, will be humanely killed. To minimise damage caused, the bacteria will be administered under anaesthesia.

Gnotobiotic mice, containing a known microbiome, do not develop the same intestinal issues as germ- free 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)?

Mouse: Mild 97%

Moderate 3%

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims to supply mice for use on other experimental projects utilising animals with altered gut microbiomes and therefore must use breeding of animals to achieve this.

Which non-animal alternatives did you consider for use in this project?

We use a range of human assays, within our laboratory, to complement this research and reduce the number of animals required. These assays use human cells from blood samples to measure the behaviour of gut bacteria.

Why were they not suitable?

Using human cells from blood samples for laboratory assays does not fully replicate the complexities of the gut barrier and the interactions which occur between gut bacteria and the immune system (white blood cells) within the gut.

Particularly in cancer research, it is impossible to measure the whole body effects of particular gut bacteria on the growing tumour within these assays.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The purpose of this service licence is to breed and supply mice for microbiome research performed on other project licences.
The number of animals are estimated based on our in-house knowledge of animals required for these projects and experience of breeding efficiency and litter sizes in germ-free mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

These animals are to be used on other project licences where the experimental design has utilised design tools such as the NC3R's Experimental Design Assistant and other computational analyses to use the minimum number of mice possible whilst maintaining the integrity of the results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are continually reviewing ways to make the breeding of our colonies more efficient in producing sufficient offspring whilst keeping the numbers of breeding pairs to a minimum.

The use of a range of human laboratory assays to complement this research will increase our understanding of the behaviour of particular bacterial species. By screening these bacteria in laboratory assays we are able to select only the best 'health associated' bacteria reducing the number of bacterial therapies to be tested in animal models.

This will subsequently help to reduce the number of animals required 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.

This project utilises breeding techniques to maintain germ-free and gnotobiotic mouse colonies. Breeding is not anticipated to cause any pain, suffering, distress or lasting harm to the animals.

In order to produce new mouse colonies a minority of germ-free mice will be given mammalian gut bacteria orally to produce gnotobiotic mice with a known gut flora (bacteria).

Why can’t you use animals that are less sentient?

Less sentient animals such as fish or frogs are not appropriate for microbiome research as these differ in gut bacterial species and are not as easily housed under germ-free conditions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During breeding of animals the age of breeders, number of litters and breeding performance will be closely monitored.

To reduce the likelihood of the enlarged caecum with age in germ-free mice breeding will be limited to 12 weeks of age for males and 18 weeks for females (if pregnant at 12 weeks).

Mice will have two daily welfare checks which include observing that animals are healthy and can move freely in every cage, have sufficient food and water and the isolator temperature/humidity readings are appropriate. These details are recorded on observation sheets within a day book for assessment of an individual animal's health status.

For the minority of mice which receive oral administration of a mammalian microbiome, this procedure has been refined to be carried out under anaesthetic to reduce the likelihood of any infection due to bacteria entering the bloodstream during the process. Post procedure checks are carried out.

Following in depth screening of the behaviour of bacterial species we will continue to work with animal care staff to assess the requirement for anaesthetic use.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure best practices we follow the updated ARRIVE guidelines [PLoS Biol 2020 18(7): e3000410]

We will follow the PREPARE guidelines to ensure all best practices are followed by researchers who use animals bred on this licence. [Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017 Aug 3.]

In addition, we have consulted the Home Office guidelines published on efficient breeding of rodents and follow updates to best practice guidelines on The Laboratory Animal Science Association, LASA website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will use the NC3Rs website to gather up to date information about advances in animal research. This information will be disseminated to anyone involved with this licence including the project licence holder (PPLH), personal licence holders working under the licence (PILs)and named animal care and welfare officers (NACWOs) and implemented where appropriate.

126. B cells and their role in type 1 diabetes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Diabetes, Immune system, B cells, Pancreas

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 this research is to understand the role that of a group of white blood cells called B cell lymphocytes play in the autoimmune disease type 1 diabetes, and specifically how these B cells interact with other immune cells, both in and outside the pancreas, to cause and perpetuate 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?

Studies have shown that B cells can play different roles in type 1 diabetes, they can both regulate or assist the development of disease. Both these roles are still not well understood. Clinical trials in targeting this cell type have shown some success, and so critical to continue this area of research. This investigation could help harness new approaches in targeting B cells for the improvement of type 1 diabetes therapy. This would

not only benefit individuals who have type 1 diabetes but also people who are at risk of developing this disease.

What outputs do you think you will see at the end of this project?

Outputs from this project will be a better understanding of the development of type 1 diabetes, including how important B lymphocytes are in this disease. This new information will help to create different strategies in trying to prevent or cure type 1 diabetes. Scientific papers on this project will be published, and these will be widely available for the community.

Who or what will benefit from these outputs, and how?

The scientific community will benefit from the new information produced, providing a basis for more studies on individuals who are at risk of developing type 1 diabetes or have already developed type 1 diabetes. These data will feed into the network of researchers, who are not only studying type 1 diabetes but also other autoimmune conditions. Type 1 diabetes is increasing in frequency, especially in children under 5 years old. In the longer term, this project will indeed help to develop treatments in order to delay, or treat, this disease, which will improve the quality of life of people living with type 1 diabetes.

How will you look to maximise the outputs of this work?

Outputs from this project will be published in peer-reviewed journals, to inform the scientific community. Also other ways of broadcasting new knowledge will be done, for example presenting work at national and international conferences with both clinical and basic scientists in attendance. Collaborating with other experts within and outside the establishment will help to maximize the outputs on this project.

Species and numbers of animals expected to be used

- Mice: 8900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 non obese diabetic mouse model has been used extensively in type 1 diabetes research. This model develops spontaneous type 1 diabetes and is very similar to human disease, therefore making it a very appropriate model to use. Adult mice of a certain age must be used in this project because immune cells that attack the cells in the pancreas will only be there during adult life, and these cells become more in number as mice age. We are unable to access these cells in the pancreas of humans, and unfortunately access to most tissue is available only post-mortem. We can only manipulate immune cells in models of diabetes, this allows us to dissect parts of the immune system without other complex interactions.

Typically, what will be done to an animal used in your project?

Many of the animals will not experience any adverse effects. However, some animals may

experience minor discomfort when an injection is given. A small number of mice will be given multiple injections, in certain experiments. However, the injection is very quick and sedation would cause more harm or distress to the animal. Mice will be injected with cells or substances that are either therapeutic or cause disease, and then monitored daily. A small proportion of animals may undergo metobolic testing, which can involve a short period of time without food (but with access to water) before being injected with substances such as glucose. Here, mice will be measured for levels of blood glucose (a very small drop of blood is collected from the tail) before and after injection.

Some animals may develop signs of diabetes, which means they will experience thirst and pass urine more frequently. Mice that have a positive test for glucose in their urine, will undergo a blood glucose test to confirm diabetes onset. Before animals have significant illness or weight loss they will be humanely killed. To check on the overall health of the animal and any signs of diabetes mice will be weighed and checked for any signs of ill-health.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that develop diabetes may start to lose weight, and will be killed humanely before any significant weight loss. Mice will be killed no later than 2 weeks after diabetes development.

Expected severity categories and the 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 the majority of breeding animals the expected severity will be mild, however approximately 20% may develop diabetes and therefore the expected severity will be moderate. Mice that are of an older age, or animals that are injected, are more likely to develop diabetes. We expect approximately 60-70% of these mice 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 will study the immune system in the development of disease. In humans, access to the pancreas in very difficult, and it is not possible to manipulate certain pathways or cells in the immune system. Type 1 diabetes develops due to immune cells in the pancreas attacking the insulin producing cells, which we need to control our blood sugar levels. We need to use animals that mimic this process, as this complex process cannot be reproduced in a tissue culture technique.

Which non-animal alternatives did you consider for use in this project?

Human alternatives are very limited, and do not provide access to the cells, that can be manipulated for study of the immune system. Other tissue culture techniques cannot mimic the complex immune and metabolic processes that happen in the body.

Why were they not suitable?

Alternatives are not suitable due to the fact we need to mimic the diabetes development process in order to study it.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 statistical considerations, this is based on providing sufficient information in our previous experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During experimental design, we explored techniques that would allow us to measure multiple factors in experiments. This means we use a reduced number of animals because we can study different factors, simultaneously, and avoid unnecessary repetition.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals numbers will be optimized in this project by considering sharing of tissues, when appropriate, for different experiments. Pilot studies will be performed before larger experiments carried out, which will allow statistical calculations, which will help calculate accurate group sizes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model we will use in this project is a mouse model which develops spontaneous diabetes, naturally with age (due to genetics) and this is very similar to humans. As these mice develop diabetes naturally, the onset of diabetes is gradual which allows us to take significant steps to minimize any harm to animals. We also do not need to induce disease, and therefore less invasive. Many animals will not experience any adverse effects, or have the chance to develop clinical signs of diabetes. In most circumstances, we can study the development of diabetes in this mouse, without having to wait for mice to have any signs of diabetes.

Why can’t you use animals that are less sentient?

It is important that our research mimics human type 1 diabetes, in order to have any translational success. The immune system and pancreatic tissue are very similar between man and mouse, and so mice make a good model for human disease. Animals that are less sentient like invertebrates or fish, compared to a mouse, do not provide the correct pancreatic structure or the complex immune processes that are involved in type 1 diabetes development.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be monitored weekly for diabetes from the age of 12 weeks. If a mouse has been positive for glucose in the urine, we can increase our monitoring of the mouse to ensure no distress to the animal. If a mouse develops diabetes, this results in increased urination, and so we can change bedding more frequently or provide wet mashed food, if required, and so improve their housing and husbandry conditions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the PREPARE guidelines (Planning Research and Experimental Procedures on Animals: Recommendation for Excellence) and ARRIVE guidelines when publishing animal studies.

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, by regularly informing ourselves of updates by using the NC3Rs website, this will help advise us of any changes in policy or practice.

127. New Treatments for Organ Injury after Sepsis and Trauma

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

sepsis, COVID-19, trauma, therapy, organ failure

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to gain a better insight into the causes of organ injury (e.g. heart, lung, kidney) after infections (e.g. sepsis or COVID-19) or trauma with the hope to find 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?

Many people in the intensive care units of our hospitals die from the consequences of sepsis, COVID- 19 or trauma every day. The cause of death is in many cases the failure of

one or more key organs (including the heart, lung, kidney) to function properly. Although there are some treatments which support a failing organ (e.g. ventilation of the lung, dialysis), there are currently no specific treatments to prevent or reduce organ injury in these conditions. We are trying to understand the specific causes that drive organ failure with the hope to develop new therapies to prevent or reduce the organ failure in sepsis, COVID-19 and trauma/blood loss. Ultimately, we hope that our discoveries, as in the past, will lead to clinical trials that evaluate new treatments in patients with sepsis/COVID-19 or trauma and blood loss and ultimately will improve patient care and reduce mortality.

What outputs do you think you will see at the end of this project?

Whenever possible, the research data arising from this project will be published in high-ranking, peer- reviewed scientific journals (ideally open-access journals) to allow for the world-wide distribution of our findings. We will also aim to publish negative findings. Data generated under the previous home office license have resulted in the publication of more than 60 scientific articles and, since 2015, our publications have been cited more than 6,000 times. We have and will also report our data at national and international scientific meetings.

Who or what will benefit from these outputs, and how?

The reporting of our research in publications and at scientific meetings (dissemination of knowledge) will not only aid the research projects of other researchers, but hopefully reduce the overall number of animal experiments (due to repetition).

Most notably, we hope that, in the longer term, our discoveries will inspire clinical trials in patients with sepsis, COVID-19 or trauma in which new treatments that we have discovered will be evaluated. The overarching aim of our work is to benefit patients with sepsis, COVID-19 and trauma by reducing the degree of their illness and also the likelihood that they die from these severe conditions. Indeed, data generated under the previous home office project license also have resulted in a discovery that was translated into a clinical trial for patients with trauma and major blood loss. Specifically, our data have importantly contributed to the development of the preclinical dossier that was submitted and approved by the regulatory authorities (MHRA).

How will you look to maximise the outputs of this work?

We frequently collaborate with other scientists in our own organisation, but also in the UK and Europe, to maximise the information gained from the clinically relevant animal experiments that we have developed and refined over many years. We have (and will) provide colleagues with organ biopsy and blood/serum samples to allow for further analysis which included an extensive assessment of the metabolic state of the animal (called metabolomics) and an assessment of many biomarkers.

The reporting of our research in publications and at scientific meetings (dissemination of knowledge) will not only aid the research projects of other researchers, but hopefully reduce the overall number of animal experiments (due to repetition). We also report unsuccessful approaches in publications.

Species and numbers of animals expected to be used

- Mice: 9000

- 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.

The proposed project wishes to gain a better understanding of the mechanisms underlying the organ dysfunction caused by conditions such as sepsis, COVID-19 or blood loss as a result of trauma with the aim to find new treatments. Adult mice and rats are most suitable for these investigations, as their responses to these conditions are similar to those of humans, and we can provide similar basic, treatment care (for example antibiotics) in rodents to those used in humans. In mice and rats, we can also measure clinically relevant endpoints and biomarkers of organ dysfunction that are also used in human studies (e.g., imaging of the heart with ultrasound). Lower species are not suitable to achieve this aim. In the mouse models, we will also be able to use genetically altered animals to investigate the specific role of a protein in disease, which helps us to find new drug targets. The replacement of these animal models with alternatives (computer-modelling of disease, cell culture studies) is not possible due to the complex nature of the diseases investigated here.

Typically, what will be done to an animal used in your project?

Anaesthetized animals will undergo a specific, abdominal surgery, which first cause a local infection and then sepsis (blood poisoning), which ultimately leads to organ dysfunction of, for example, the heart and kidneys within 24 h. All animals will receive intensive monitoring and appropriate care including antibiotics. Some animals will receive treatments that are expected to reduce organ dysfunction. At 24 h, the dysfunction of the heart will be assessed by imaging and all animals will be humanely killed to obtain tissue biopsies and blood samples for further investigation.

Alternatively: In our COVID-19 sepsis model, animals do not undergo surgery, but will receive injections of agents (for example the SARS-CoV2 spike protein) to cause inflammation and organ dysfunction within 24 h. Some animals will receive treatments that are expected to reduce organ dysfunction. At 24 h, the dysfunction of the heart will be assessed by imaging and all animals will be humanely killed to obtain tissue biopsies and blood samples for further investigation.

Alternatively: In our trauma-blood loss models, anaesthetized animals will undergo a specific surgery to cause severe blood loss, which ultimately leads to organ injury of, for example, of the heart, lung and kidneys within a few hours. All animals will receive intensive monitoring some animals will receive treatments that are expected to reduce organ dysfunction. All animals remain unconscious and will be humanely killed at the end of the experiment to obtain tissue biopsies and blood samples for further investigation.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals are expected to recover uneventfully from the sepsis-surgery or injections of proteins to cause a COVID-like state. No death is predicted by these protocols, but some

animals are likely to experience discomfort and pain of the mild to moderate severity for a short time after surgery or injections of agents that cause inflammation. These adverse effects will be limited using pain killers, antibiotics and fluids and all animals will be carefully monitored. In our models of trauma and blood loss, all procedures will be carried out under terminal anaesthesia and the animals do not recover 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)?

The maximal severity of any of the proposed procedure is 'moderate' and approximately 15-30% of all animals will experience this degree of severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed project aims to develop a new treatment to prevent or reduce the organ failure caused by sepsis, COVID-19 or trauma and blood loss. In order to evaluate therapeutic effects of the treatment, measurements of the degree of organ injury and dysfunction after the treatment are essential. The process that leads to the development of organ failure is extremely complicated and involves many cell types (both locally resident in an organ and recruited) as well as many molecules and signalling pathways, which cannot be mimicked without the use of animals.

This project includes practical investigations using cultured cells, isolated organs, and in vivo studies using whole animals. For both ethical and economic reasons, animal studies are only pursued once sufficient in vitro data regarding a hypothesis has been collected justifying further in vivo investigation. Prior to performing any in vivo experiments, we will perform a thorough literature review to prevent unnecessary duplication of animal work that has already been done and to gain insight into any potential adverse effects of an intervention.

Whenever it is possible for experiments on animals to be avoided, then alternative experiments are suggested and performed (see below). The employment of animals of a lower phylogenetic scale in sepsis research is still in its infancy, but may become of interest in the future. However, the-wide ranging and multi-system effects of both sepsis and trauma, much of which remains poorly understood, means that the total replacement of animal experiments with other methods would not be feasible at the present.

Although animal experiments cannot be replaced to achieve the stated goals of this project, we have made every effort to reduce the number of animals used to achieve a maximum effect. We have considered each procedure in our application with a view to

developing strategies, which will reduce the number of animals used to obtain the same amount of information, or maximising the information obtained per animal and thus limiting or avoiding the subsequent use of additional animals. For example, when performing procedures, we will alert colleagues when the animals will be killed so that other researchers may be able to make use of other organs or tissues from the same animal.

Which non-animal alternatives did you consider for use in this project?

We can, and have, used in-silico (computer-algorithm) techniques (based on machine-learning techniques that used data generated by in vivo experiments during the previous PIL) to model the progression of the organ dysfunction associated by sepsis. This has given valuable insights into the timing of any potential interventions with treatments (drugs).

We are using cell culture techniques to investigate the suitability of compounds in our models and complement data gathered in animals. Specifically, immortalised cell lines from rats and humans may be used to identify suitable therapeutic strategies, which are then evaluated in animal studies. Interventions that were ineffective (or largely ineffective) in cell culture studies were not evaluated in vivo to reduce the number of animal experiments.

In the past, we have used isolated-perfused hearts obtained from animals (after schedule 1 killing) to confirm that the heart failure in sepsis is, indeed, secondary to the reduced pump function of the heart. These experiments are no longer necessary.
Why were they not suitable?

Although these techniques are able to refine and reduce the number of animal experiments needed to achieve a scientific goal, they cannot replace all animal experiments in their entirety with alternatives as we investigate multiple organ systems. The interaction of these organ systems is very complex, and we have observed many interactions of local and circulating cells and proteins (cytokines/chemokines) that drive the organ dysfunction in sepsis and trauma. In addition, the discovery of biomarkers that predict the failure of an organ is not possible in cell studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have significant experience with the proposed animal models and, over the years, have refined these models to minimise the variability of the key endpoints. This helps us to minimise the number of animals needed to answer a specific scientific question. The numbers of animals needed to successfully carry out the proposed project are estimates based on past experience with the same animal models. They constitute maximum numbers, and it is likely that the numbers needed are lower than estimated here, we will continue to refine our animal models and replace some aspects of our research by utilising computer-models to guide us.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the Experimental Design Assistant (EDA) of the NC3Rs to guide our experimental design. The EDA may aid good experimental design and can minimise animal use in two ways: A) by accounting for the influence of variables and addressing sources of bias, an adequately designed experiment will yield robust and reproducible data, ensuring that the data from every animal is utilised to its full potential. B) An efficient use of statistics can reduce the number of animals required and maximise the information obtained per experiment. More complex designs, for example, can help researchers identify factors which influence the experimental results, providing more information about the model they are using. The design of individual experiments generally involves factorial designs, which maximizes the information obtained from the minimum resource. Most of our measures are quantitative, and a statistical analysis will be performed.

Breeding approach: Whenever possible, we will obtain specific animal strains (e.g., genetically- modified strains) in small numbers from collaborators. It is not our intention to breed large number of animals for this project. If a breeding approach is chosen for a specific experimental cohort, we will take a very targeted approach to ensure a close match between breeding and experimental requirements with minimal waste in line with the guidelines relating to 'Colony management best practice' provided by the N3CR. As suggested by the NC3R, we will follow the advice outlines in 'Breeding strategies for maintaining colonies of laboratory mice' from the Jackson Laboratory.

We employ experienced research scientists, who already have significant expertise in the proposed models, to ensure the highest quality of our research, while minimising the numbers of animals needed to achieve this.

Good practice should prevent, or at least minimise the introduction of bias into the experiments. This will, in turn, help significant reduction of the animal numbers used in this project. Accordingly, individual experiments will, whenever possible, include randomisation of treatment or control, blinded allocation of treatment, blinded assessment of outcome, clearly defined inclusion and exclusion criteria, sample size calculations before the experiment (Power-Calculations), monitoring and controlling of blood pressure (when possible) and temperature during surgery, and stable depth of anaesthesia.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have and will (during this project) increasingly use in-silico (computer-algorithm) techniques (based on machine-learning techniques that used data generated by in vivo experiments during the previous PIL) to model the progression of the inflammation and organ dysfunction associated with sepsis and trauma. This has given valuable insights into the optimal timing of any potential treatment with drugs, and this not only increased our success rate, but also reduced the number of experiments needed to achieve a specific goal.

We also continue to use cell culture techniques to investigate the pathways that we wish to target to reduce inflammation and organ dysfunction. For example, we use heart cell lines to gain a better insight into the mechanism that drive the heart dysfunction in sepsis. Once identified in cells, they can then be 'treated' in cells before we move to an animal experiment. This has also reduced the number of animal experiments needed to achieve a

specific goal.

A pilot, or feasibility study, is a small experiment designed to test logistics and gather information prior to a larger study, to improve the latter's quality and efficiency. It can reveal deficiencies in the design of a proposed experiment or procedure, and these can then be addressed before animals, time and resources are expended on large scale studies. A good research strategy requires careful planning, and a pilot study will often be a part of this strategy.

We routinely share tissues from our well-established and refined animal models with other investigators in our organisation, but also in the UK and Europe.

Our research questions are often informed by clinical data and we ensure that a drug target is relevant in humans (for example a biomarker is increased in humans with sepsis/trauma), before we investigate the same target 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.

In this project, we are using mouse and rat models of injury and severe blood loss and mouse models of systemic infection (sepsis) caused by bacteria and/or viruses (COVID-19). All animals models with trauma/injury and blood loss are under terminal anaesthesia to minimise suffering of the animals.

Patients with sepsis die from multiple organ failure and animal models of sepsis need to reflect this to be come clinically relevant and to aid the evaluation of novel therapies. In mice, we cause sepsis secondary to a surgical procedure, but all animals (like patients) are carefully monitored and receive supportive therapies including antibiotics, pain killers and fluids. During our previous license, we have established that a period of 24 h is sufficient to cause a dysfunction of the heart and kidney that is reproducible. Thus, we have refined our model and limited the experimental period to 24 h. The current COVID-19 pandemic has highlighted the need to understand the mechanisms, which drive organ dysfunction in infections with the SARS-CoV-2 virus. During this license, we are working to use viral proteins (e.g., spike protein) to develop a model of SARS in the mouse in order to find new therapies for the organ dysfunction in patients with COVID-19.

Why can't you use animals that are less sentient?

The employment of animals of a lower phylogenetic scale in sepsis research is still in its infancy, but may become of interest in the future. However, the wide ranging and multi-system effects of both sepsis and trauma, much of which remains poorly understood, means that the total replacement of animal experiments with other methods would not be feasible at the present.

Although all animals in our trauma-project will remain under terminal anaesthesia, this is not possible for our sepsis studies, as the organ dysfunction in sepsis only occurs 15 to 24 h after onset of the infection and anaesthesia for these prolonged periods is not feasible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our long-term ambition is to transfer our in vivo work from rats to mice, for this reason we have been developing these models in mice and so have reduced the usage of rats. To further this end, we are increasingly using genetically-modified mouse strains allowing us to target our experiments better, using smaller and fewer experiments and consequently resulting in fewer animals used overall.

The procedures we choose to use are those we are most experienced with and find successful, however, we have introduced some additional procedures which are designed specifically at refinement. We have reduced the experimental period of our sepsis model from an initial 3-4 days to 24
h. We have now an established model of sepsis, with minimal variability and very good monitoring and post-operative care and pain management. Our aim to establish a model of COVID-19 (viral sepsis) will be guided by our significant experience in sepsis model and we hope to establish an acute model of inflammation and organ dysfunction driven by viral proteins.

Most of our procedures are well established and refined. However, we will continue our effort to further refine the procedures to minimise animal suffering. This may be achieved using newly-introduced, more effective analgesic regimen and/or antibiotics (discussed with the NVS), less-invasive surgery, including minimising the incision and reducing the surgical time, improved post-operative observation procedures, minimally invasive ways to assess the progression of organ injury/dysfunction, including the use of advanced imaging modalities.

Our research has contributed to the development of guidelines aimed at the refinement of research strategies that are mentioned on the website of the N3CR (see above).

Breeding approach: Whenever possible, we will obtain specific animal strains (e.g. genetically-modified strains) in small numbers from collaborators. It is not our intention to breed large number of animals and setting up large colonies for this project. If a breeding approach is chosen for a specific experimental cohort, we will take a very targeted approach to ensure a close match between breeding and experimental requirements with minimal waste in line with the guidelines relating to 'Colony management best practice' provided by the N3CR.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We regularly review and discuss any new HO guidelines relating to animal experiments, the NC3R homepage/newsletter.

Based on our research, we have also been invited to contribute to the development of guidelines that aim to improve the development or refinement of animal models that are clinically relevant, but humane animal in the areas of sepsis and trauma. We also

collaborate with colleagues from the RSPCA and the HO and, more recently, a panel of experts of the European and the US Shock Societies. Indeed, we are proud that the website of the N3CR (if you search for the term 'sepsis') refers to a lecture relating to the Minimal Quality Thresholds for Preclinical Sepsis Studies. This lecture is based on a series of publications endorsed by the World Federation of Shock Societies (including the European Shock Society) and the European Society of Critical Care Medicine om which the applicant is one of the (31) co-authors from 13 nations.

We follow the ARRIVE and PREPARE guidelines as well as the recommendations published as a joint effort of the European and US Shock Societies entitled Minimum Quality Threshold in Preclinical
Sepsis Studies'

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stay informed about the latest advances in the 3Rs through information/newsletters from the Home Office and attendance at relevant academic conferences (e.g., the PAN-LONDON 3R Symposium held online in March 2021) and regular literature searches. Indeed, I have subscribed to the NC3R newsletter, and the research team regularly discusses the N3CR updates in our lab-meetings. Indeed, we have recently been complemented by N3CR on our publications in these areas of research and jointly evaluated the impact of our guidelines on the sepsis research community. Our research team has been instrumental in setting up international and national collaborations that contribute to the development of guidelines on refinement of sepsis and trauma models and their care and support. Such guidelines are now endorsed by the World Federation of Shock Societies (including the European Shock Society) and the European Society of Critical Care Medicine and, indeed, mentioned on the website of the N3CR.

128. Creating new targeted therapies for cancer and wound healing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, Wound healing,

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 this project is to cancer and would healing therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer therapy and wound healing therapy are sometimes toxic and don’t always produce the intended therapeutic outcome. Therefore, there are many drugs which, although they look promising in the laboratory, cannot move further to pre-clinical studies and/or to clinical trials. We aim to solve those issues using our unique technologies.

What outputs do you think you will see at the end of this project?
This project will generate new knowledge through the publication of work in peer reviewed journals. The knowledge will include design principles of next-generation therapeutics that potentially enable to treat refractory diseases (those which don't respond to treatment) while minimizing side effects. We also expect to uncover some aspects of disordered microenvironment of diseases and suggest solutions.

Secondly, we will develop molecular engineering technologies that hold translational potential to the clinic. We aim to provide medical benefits to society. We hope newly developed therapies will eventually save the lives of some people.

Who or what will benefit from these outputs, and how?

The knowledge obtained through this project will be available as forms of research publications and scientific presentations, giving benefits to the research community. Patients will benefit through the development of new treatments for cancer and would healing.

How will you look to maximise the outputs of this work?

Collaboration: We will spread our findings and technologies through collaboration with industry and academics.

Dissemination of new knowledge: we will present our findings at conferences and will publish our work in peer reviewed journals.

Species and numbers of animals expected to be used

- Mice: 5500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

For cancer experiments, we will treat cancer-bearing mice through drugs. Mice will be typically injected drugs a few times. For wound healing experiments, back skin of the mice will be punched under painkiller and anesthesia. Mice will receive drugs through injections. Mice will be monitored until mice will reach humane endpoints. Mice may be bled to check their health status and body weight and their appearance will be checked. These are typically up to three months.

Explain why you are using these types of animals and your choice of life stages.

Mice are the smallest and least sentient type of animals that have established cancer and wound- healing models. Practically, both cancer and wound-healing treatments using mice are well-accepted widely, also, many research tools are available. Mice and humans share similar characteristics regarding these diseases.

We will use animals that more than 8 weeks, which immunologically matured and the life stage is more relevant to human diseases.

Typically, what will be done to an animal used in your project?

In cancer studies, a typical animal will be implanted with cancer cells through injection. An implanted tumour will be detectable within 1 week, at which time the animal will receive injections of therapeutics to evaluate their effectiveness and side effects.

In wound healing experiments, a typical animal will receive an injection of painkiller before wounds are made under anaesthesia. The animal will receive administration of therapeutics, and wound closure will be monitored..

What are the expected impacts and/or adverse effects for the animals during your project?

Injection may cause swelling or inflammation at the injection site. The most common side effect of chemotherapeutic agents are decrease of white blood cells or gastro-intestinal disturbance. The most common side effects of protein-therapeutics and therapeutic cells are transient liver damage and increase in serum cytokine 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)?

In this project, we expect that 90% of the animals will experience moderate severity, and rest of the animals will experience mild or no severity. There will be no proportion differences of severity categories among animal types.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We will use cells and experiments with tissue-mimicking materials in addition to animals. We will screen novel compounds/drugs using laboratory methods prior to using them in animals. Nonetheless, we need to use animals because cell cultures and tissue-mimicking materials used in the laboratory are neither good models of diseases nor good platforms for evaluating cell-protein interactions with biological tissue. An extensive literature search to find non-animal models to study the effects of drug discovery on cancer and wound healing rendered negative results. Current cell culture models cannot model many aspects of complex microenvironments . We require animal models because the human body system is too complex to mimic in a culture dish. Also, the tumour development models in the laboratory have limitations in mimicking circumstances in live animals.

Which non-animal alternatives did you consider for use in this project?

Cell lines such as cancer cells, immune cells, and endothelial cells in culture.

Why were they not suitable?

The body has complicated structure of biological interactions and flow. Cells are good to test very simple assessment, but they do not reflect the human body. Thus, experiments using cells are not enough to assure the safety and efficacy of our newly developed drug and biological discovery.

Only a higher mammalian organism can be used to reproduce a 3D tissue microenvironment and pharmacological analysis necessary for our research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have calculated how many experiments we will do and how many animals we will use based on experience and relevant literature..

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to ensure that the use of animals under this license is reduced to the minimum required to achieve significant results the following measures have been taken:

Computer-based and laboratory methodologies will be extensively used prior to experiments using live animals. Longitudinal studies allow for overall reduced number of animals to be used. We will optimize the experimental conditions of our experiments in animals which reduces the potential number of animals required for meaningful results.

When several parameters or markers can be studied at once, several laboratory and live animal techniques will be applied to maximise the amount of information gained from the animal.

Experiments will also be designed so that we can publish to the NC3Rs ARRIVE guidelines. Where relevant, we will carefully consider randomisation, blinding, and the sample size of our test groups. Studies may be randomised to reduce bias, confounding factors, and chance events. Where relevant, we will apply appropriate blinding, sample size calculations, and statistical methods. In some studies, such as dose optimisation and tolerability studies, we will use fewer animals in a separate study 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?

We will use power analysis if the similar experiments are previously reported. Where necessary, we will perform pilot experiments using small number of mice to estimate the

variance and then we will calculate how many mice we should use for the experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse cancer and wound healing models. Compared to large animals, mouse disease models are more established and quicker. Therefore, the use of mouse can reduce pain, suffering, distress and lasting harm. We will humanely kill mice when mice show the sign of harm and at the end of the procedure to reduce pain.

Why can't you use animals that are less sentient?

Mice were chosen because they are the lowest animals on the evolutionary tree for which many suitable and established models of cancer and wound healing are available. Especially, immune system and cancer development, wound healing process are similar between mice and human. Non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) or less sentient animals (e.g. zebrafish) cannot be used due to lack of suitable disease models relevant to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Cancer models. A limited number of mice will be used to characterise the tumour traits (e.g. expected growth rate) prior to their use in larger studies. This is to determine whether the patterns of growth are reproducible, show any adverse effects associated with tumour progression, and identify humane endpoints. We will select cancer models (e.g. cell line, injection route, etc.) that are appropriate for the scientific objective while minimising the stress to the animal. Mice will be monitored to check that they are not suffering too much.

Wound healing models. Pain relief will be given prior to wounds being administered and this will be performed under anaesthesia. Mice will be monitored to check that they are not suffering too much.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ASPA guidance and the LASA guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will search relevant papers every month. Especially, papers that describe in vitro cancer models and wound healing models in animals will be searched online. We will liaise

with the NACWO/NVS. We will regularly check any updates on the website of NC3Rs as well.

129. Assessment of dermal injury and repair

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

skin, wound, venous ulcers, chronic, Injury

Animal types	Life stages
Pigs	juvenile
Minipigs	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall objective of this research project is to develop treatments (agents/dressings/devices) which, when used as a treatment on normal or recalcitrant dermal wounds, will be beneficial to the repair 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?

Injury to the skin (epidermis and underlying dermis) is a common every day incident. The injury may be a minor or major trauma, or may occur as a consequence of another underlying disease. During normal wound healing, dressings are usually applied to: I) manage exudates or wound fluid; II) influence moisture levels; and III) provide some protective barrier. The wound generally repairs both rapidly and successfully. Although

acute dermal injuries usually repair rapidly with little or no problems, the situation with chronic dermal repair is very different. Wounds that have been compromised (for example through infection), or are recalcitrant (delayed healing) due to underlying illness or treatment can take many months to heal. Such wounds are seen in patients with conditions such as compromised venous vascular supply (varicose veins, heart disease), and in immunosuppressed and diabetic subjects. Other poor healing situations arise when there is copious eschar (dry, black, hard necrotic tissue) formation or excessive scarring.

This clinical problem is extensive. Data from 56 (29%) of 192 WHO member states estimates that 234.2 million major surgical procedures are undertaken, annually, worldwide (Weiser, T.G. et al; 2008; An estimation of the global volume of surgery: a modelling strategy based on available data; The Lancet, 372: 139-144). In the U.S.A. alone chronic wounds affect around 6.5 million patients whilst venous ulcers cause the loss of 2 million working days per year, accounting to 70-90% of ulcers found on the lower leg. The prevalence of venous ulcers in the U.S. is approximately 600,000 annually (Sen, C.K. et al; Human skin wounds: a major and snowballing threat to public health and the economy; 2009; Wound Repair Regen, 17(6): 763-771). In venous stasis 20% of chronic wounds (over 2-3 weeks) and 40-50% of diabetic chronic wounds do not repair. It is claimed that more than US$25 billion is spent annually on the treatment of chronic wounds.

Thus there is a real need to provide better treatments for the healing of recalcitrant and poorly healed wounds and lower levels of scarring.

What outputs do you think you will see at the end of this project?

The primary output of this project is to develop new products/methods to treat dermal wounds and/or enhance dermal repair using existing experimental in-vivo models.

Data gathered from in-vivo studies will allow us to assess the benefits of the dressing/treatment in the management and promotion of the injury and repair processes. Should a particular treatment be successful then the data will also provide confidence for progression to clinical trials, facilitating the development of innovative products, which will improve patient outcomes.

The underlying biology of repair will be assessed biochemically, histologically and via gross pathology observations to gain a greater understanding of dermal healing. Identification of potential treatments and their benefit can then be understood more fully. Additionally, this will help gather scientific knowledge and generate publications to better the wider scientific community.

Who or what will benefit from these outputs, and how?

The primary output will be to develop dressings, devices and treatments for use in humans where the range and types of wounds to be treated will vary greatly in size, depth and aetiology. For example, chronic recalcitrant wounds will be treated with dressings (e.g. negative pressure therapies) that will promote wound repair, such as in chronic venous ulcers.

Our long-term goals are to develop dressings/devices, that may contain active agents (e.g. cells, growth factors, matrix, or possibly pharmacologically active components), and negative pressure therapy wound treatments that promote repair. If this approach is

successful it will make a very large impact on wound healing in human patients with recalcitrant wounds.

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 products/treatments for dermal repair.

Product development may also involve the support of either academics or clinicians, which will assist with knowledge transfer. The outputs from these studies will be archived and may be used by the research team initiating the line of work, as well as other research teams, as historical data for internal learning. Best practice may also be disseminated via, for example, the I.A.T. animal welfare discussion group, upon request.

Species and numbers of animals expected to be used

- Pigs: 200
- Minipigs: 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 domestic pig has morphological and functional characteristics which are comparable to human skin. Porcine epidermis is 70-140 µm thick, (human epidermis is 50-120 µm) and some breeds are only slightly pigmented making observation of inflammation easier. The dermis is similar and the vascularisation of the lower region of the hair follicles corresponds to humans. Perhaps most importantly to our wound healing studies is that the proliferation rate of porcine epidermis shows distinct parallels to man (Meyer W & Neurand, K; 1978, Current Problems in Dermatology 7 39-57) and the wounds do not repair by excessive contraction (as seen in the rat model) which again is comparable to human dermal repair. The research team have used the porcine model for dermal repair for over 35 years in the assessment of new products, dressings and materials on excisional, eschar forming, incisional and intradermal wounds. The porcine model is used where new products and dressings are assessed and where the requirements of efficacy needs comparison to human skin.

Juvenile (over 10-weeks of age, NVS advice at AWERB) and adult pigs will be used as this presents a larger surface area of skin that will permit the application of full size dressings designed for the human market.

Typically, what will be done to an animal used in your project?

Animals will enter the facility and undergo a period of acclimatisation, lasting a minimum of one week but is likely to be for a period of three to four weeks. Pigs will be either group or singly housed, depending upon the animal's temperament. Group housing will be encouraged but if there are signs of fighting then certain individuals may be singly housed,

although snout touching will be possible; however see below for play periods. There may be the necessity to singly house all animals overnight. During the acclimatisation (and post-surgery) period animals will receive environmental enrichment (balls, toys and chews etc.) in addition to regular staff contact. Play periods will be initiated, either singly or in groups. Individuals that have been singly housed due to temperament issues may receive group play whilst under staff supervision. During this time any animal training may be undertaken such as becoming accustomed to non-invasive techniques, e.g. handling, weighing crates and possibly the wearing of jackets, slings and clicker training.

After acclimatisation, each pig will undergo general anaesthesia via intra-muscular, intra-venous and inhalation routes of administration. The combination of these routes of administration may vary depending upon the advice of the NVS. Each pig will then receive a maximum of twelve (domestic pig) or eight (minipig) 2cm2 or 2cm diameter wounds (six per flank domestic pigs and 4 per flank minipigs) . These may be partial thickness excisional wounds (removing only the top epithelial layer) or full- thickness excisional wounds through the epidermis and dermal layers, but not into the underlying muscle; although the panniculus carnosus may be removed. The panniculus carnosus is a thin striated muscular layer, closely attached to the skin and fascia of most mammals, with a function for skin contraction and twitching.

Following wounding, each site will be dressed with either a test dressing or a control. These dressings in turn will be covered with a protective dressing layer or a custom made protective jacket. Each pig will receive an injectable analgesic, such as buprenorphine, prior to recovery; this will usually be via the intra-muscular route but will also be dependent upon the advice of the NVS. Analgesia will be administered for a minimum of 48-hours post-surgery. The surgical procedure is expected to last about 1 1/2 to 2-hours.

Pigs will then be returned to their holding pens and observed until full recovery. Pigs will be singly housed until fully recovered. Group housing will be encouraged as discussed above for the acclimatisation period. However, if there are signs of pen mates interfering with dressings then single housing may be required, although snout touching will still be possible, with supervised group social/play periods. Although damaged dressings can be replaced, it is essential that the wounds themselves are not damaged further as this may compromise the integrity of the study and the potential of unnecessary animal welfare issues.

At set time-points after surgery (not exceeding 12-weeks post-surgery and a maximum of 15 episodes), the pigs may be re-anaesthetised, as above, to permit observations of the wound healing, dressing performance and dressing replacement. Following these observations each wound will be re-dressed as above.

Each study may last between 2 to 5-weeks, with a maximum of 12-weeks duration.

At the final observation time-point each pig will be anaesthetised as above. Following the final set of wound observations the pig will remain under anaesthesia and then euthanised using a Schedule-1 method (pentobarbitone overdose). Once the pig has been euthanised and death confirmed the wound sites will be excised for histological, micro CT or biochemical analysis. Photographs obtained during the wound observation periods will also be analysed to determine, for example, the area of the wound and the rate of wound contraction and re-epithelialisation.

Body tissues and organs not required for the current study may be collected following

death, stored and utilised in future ex-vivo / cadaveric studies.

What are the expected impacts and/or adverse effects for the animals during your project?

On arrival the animals will be subjected to a new environment and its associated stress (in addition to transportation stress), hence the acclimatisation period discussed above.

All pigs will receive injections at various stages in the study period. Pain associated with injections will only be transient and mild during the actual procedure. However, the associated stress may last longer, for example up to half an hour. This length of time may be reduced by the pig training received during the acclimatisation period, for example staff contact/handling and inducement using treats.

General anaesthetic deaths and insurmountable technical operative problems should be very rare. However, if this should occur then the animal will remain under anaesthesia and not allowed to recover. As such it will not be subjected to any unnecessary pain, psychological stress and discomfort.

However, it is expected that pigs will be quieter and subdued post-surgery/anaesthesia and this usually lasts for a couple of hours post-surgery/anaesthesia. However, it is expected that the pig's normal behaviour will be seen by the end of the working day. In some cases pigs may become more aggressive and irritable with constant handling and injections. Again the pig training period during the acclimatisation period may help offset this.

The number of subsequent anaesthetic episodes for restraint purposes will normally not exceed 7 in the initial two weeks; this will be for the purpose of conducting post-surgery wound observations.
Additional episodes during this time may occur as a result of the animal disrupting/removing the dressing which will require replacing. However, this may be achieved using sedation rather than full general anaesthesia. Pig training during the acclimatisation period may permit additional wound observations without anaesthesia but this will be dependent upon the animal's temperament and response to the training regime.

Pain and post-operative discomfort will be an inevitable consequence of the wounding procedure but this will be controlled as much as possible via the use of analgesics, such as buprenorphine, for a minimum period of 48-hours. Only mild pain should be experienced beyond 48-hours but if this is not the case then additional analgesia will be administered under the guidance of the NVS. As the wounds heal itching may become apparent with signs of the pig rubbing its flanks on the wall of the pen. It is expected that these signs may last up to 10-days post-surgery. All animals are expected to remain healthy throughout the study.

Significant surgical sepsis is unlikely but if the condition is suspected (appearance of general malaise, loss of appetite and depressed demeanour, redness, swelling of wound) then the animal will be promptly and humanely euthanised irrespective of the requirements of the study.

Weight loss is not expected and in our experience pigs feed readily immediately after surgery. However, if weight loss is apparent then the NVS will be notified if a 10% loss in

weight is seen.

Any acute local or systemic reactions to the dressings/treatments are very unlikely but if the problem arises then the animal will be promptly and humanely euthanised irrespective of the requirements of the study. Some studies may be designed to specifically investigate the presence of any local or systemic reactions; however, previous in-vitro and in-vivo rabbit irritation studies (conducted under a different project licence) should limit the extent of any reactions 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)?

All animals, both domestic pigs and minipigs, will receive anaesthesia and wounds and as such 100% of the animals will experience a moderate severity banding. Due to the wound observation periods put in place and pen side care/observations it is not envisaged that this severity banding will be exceeded. If there are any indications that this eventuality may occur then the animal will be promptly and humanely euthanised irrespective of the requirements of the study.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Dermal wound healing is a complex, well-orchestrated physiological process involving signaling cascades and cellular repair mechanisms that cannot be studied by in-vitro and ex-vivo cadaveric studies alone where these complex interaction are impossible to truly replicate. Consequently, dermal wound healing can only be demonstrated with the help of animal models, i.e. no in-vitro, ex-vivo or computer simulations can mimic the complexity of an in-vivo environment sufficiently or predict clinical efficacy. Dermal wounds are typically caused by various conditions, (e.g. pressure ulcers, Iatrogenic consequences (side-effects) of surgery and trauma etc.), which challenges clinicians and biomedical scientists alike. The in-vivo models used under this licence attempt to reflect the physiology of the clinical scenario in humans.

Regulatory authorities such as the MHRA (UK) and FDA (USA) will also require the testing of novel dermal repair therapies in both a small animal model (under a different Home Office project licence) and a pig model before accepting an agent for clinical trials to ensure clinical translation through assessment of appropriate efficacy and safety endpoints.

Which non-animal alternatives did you consider for use in this project?

Artificial bench top models were considered. These may consist of a plastic mould with a cavity the dimensions of the wounds in question. This cavity may be filled with saline or a gel and overlaid with the dressing in question. An inflow and outflow may be set up for liquids or gases. Ex-vivo tissue was also considered for use in a similar way as to the bench top models.

In-vitro cell cultures were considered for the assessment of the potential toxicity of test materials and devices.

Why were they not suitable?

Dermal wound healing is a complex, well-orchestrated multiple physiological process involving signaling cascades and cellular repair mechanisms that all interact. Therefore it is not possible to study these complex interactions by in-vitro and ex-vivo cadaveric studies alone. Consequently, dermal wound healing can only be fully demonstrated and clinical outcomes predicted with the help of animal models. The in-vivo models used under this licence will therefore attempt to reflect the physiology of the clinical scenario in humans.

Furthermore, regulatory authorities such as the MHRA and FDA will require evidence of in-vivo safety and efficacy data prior to clinical evaluation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 research team have estimated that about four studies per year will be required with an estimate of 6 pigs per study. This would equate to approximately 120 pigs over the course of this 5-year project licence. If spare animals are included this would be about 140 pigs over five years. 200 pigs have been stated to give some leeway, for example, if an additional study was initiated per year then this would increase the total to 175 pigs. However, it should be stated that this is a total for both domestic pig and minipig combined but as it is unknown which breed will be required for each particular study then the total has been quoted for each breed. There will not be a mix of breeds within a study.

The actual number of animals per study required will be determined from a number of sources;

The experiences gained under previous project licenses and from previous work conducted at external contract houses.

ISO standards (ISO 10993).

Statisticians will 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 AWERB will assess all protocols and experimental design prior to the start of a study, 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?

The research team will adopt multiple strategies that will help ensure that the fewest number of animals are used in the research to address the scientific questions outlined in the project. Previous studies or studies reported in the literature will be used to provide variability data to aid this process.

BIOSTATISTICS/POWER ANALYSIS: Statisticians will be consulted in the planning stage of the in-vivo studies to determine the minimum number of animals required for statistical analysis and to answer the scientific question being asked. This will reduce the numbers of animals used in total without compromising the data/information obtained. Randomisation tables will be generated for dressing application across multiple wound sites on the one animal, maximising the number of test articles that can be evaluated in each animal.
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. Typically, pilot studies to assess new technologies under this licence will consist of no more than six animals. Where there is no adequate data to power a study, a pilot study will be used to gather sufficient data to design a definitive study.

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 to minimise them. This will include some or all of, but not limited to, the following (a) using well- characterised devices 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 acclimatisation, (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.

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 and ex-vivo Testing: In-vitro cell culture and ex-vivo cadaveric models will be used for studying initial concept technology by applying controlled stimuli to relevant cell lines and and concept devices to ex-vivo tissues. Ex-vivo tissues may be collected from abattoir sources or collected from animals euthanised following completion of previous studies. These tests will help establish basic functionality of new concept devices and also to establish safety of any technology prior to any in-vivo experimentation. Thus any animals

used on a study may consequently be utilised further, after death, in follow-up refinement or new concept studies.

QUALITY ANIMALS/VETERINARY CARE/PRE-SCREENING: The loss of animals from a study and hence the loss of data, that may compromise the integrity and statistical powering of a study, can also be minimized by ensuring only healthy animals are recruited onto a study. This can be achieved by sourcing the animals from reputable specialist suppliers, health pre-screening of the animals by the NVS, providing good husbandry and post-operative care, and planning ahead so that the appropriate number of animals needed for the studies are ordered. Environmental enrichment will also be encouraged to maintain the animals psychological wellbeing and reduce stress, which in itself will reduce the variation in results and optimise the quality of the data obtained from the set number of animals recruited onto study.

PILOT 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 performance of new technologies under this licence, no more than six 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 test 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, radiography (micro CT), histology and biomechanical testing.

SHARING ANIMALS: For instance, animals euthanised by one investigator can provide tissue for use by another investigator on another licence or 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.

Domestic pigs and minipigs will be used as they have morphological and functional characteristics that are comparable to human skin. Porcine epidermis is 70-140 µm thick, (human epidermis is 50-120 µm) and some breeds are only slightly pigmented, making observation of inflammation easier. The dermis is similar and the vascularisation of the lower region of the hair follicles corresponds to humans. Perhaps most importantly to our wound healing studies, the proliferation rate of porcine epidermis and dermal contraction

rate shows distinct parallels to man.

A thorough investigation into the most relevant species (pig) for simulating dermal repair has been obtained from the knowledge gained from previous project licenses and work conducted at external contract facilities. The principal method types in this project licence application are (a) full-thickness circular or square excisional wounds, or (b) partial-thickness circular or square excisional wounds. The full-thickness circular or square excisional wounds are the most widely described and used in the literature.

These pig models and methods, in conjunction with the appropriate use of analgesic regimes, have shown that they cause the least amount of pain, suffering and distress to the animals.

INITIAL SCREENING: Rabbits are suitable for safety evaluation (biocompatibility) of technologies at an earlier stage of development, utilised under a separate Home Office licence, prior to production of devices designed for humans. There has been accrued a great deal of experience with these animal species from previous licenses, which in turn has led to refinements in surgical technique, analgesic regimes and post-surgical care.

These pig models are also accepted within ISO:10993:parts 6 and 11. The standards are internationally agreed and accepted by experts and are the models that obtain the required information causing the least possible suffering to the animal.

Why can’t you use animals that are less sentient?

Protocols within another Home Office project licence assigned to the less sentient species (rabbit) are generally intended to be used for the initial/earlier stage biocompatibility assessment of technologies tailored for enhanced dermal repair. Rats are also widely used within the literature but these tend to be used when investigating the physiological aspects of wound repair rather than the efficacy of dressings and devices. Rat wound healing is primarily driven by contraction and close at a much more rapid rate than that in pigs; usually closed in about 7 to 14 days. Pig wounds, as in humans, heal by contraction, granulation tissue formation and re-epithelialisation; the turnover time of pig epidermis, like human epidermis, is quoted as approximately 30 days. Rat size also limits their acceptance for the types of studies proposed by this licence, i.e. assessing dressings and devices of a suitable size for use in humans.

As the technology progresses through the product design control matrix, there will be a requirement to assess their safety and efficacy in animal species whose anatomy and physiology more closely represents the intended use in humans. These animals (pigs) tend to be of higher sentience.

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, device 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 macro-environment, (f) increased monitoring and surveillance, (g) refinement in surgical techniques, e.g. modern anaesthetic monitoring equipment, and aseptic techniques to reduce the risk of infection, (h) up to date pain management (anaesthesia, analgesia and sedatives) to minimise pain, and (i) refinements in post-operative care and recovery.

SOP's will also be regularly updated and documented within the facility Quality Management System, which is accredited to ISO9001 and Good Laboratory Practice (GLP) compliant. 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 welfare, minimise 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) the AWERB with an advisory function on ethical matters, (b) UK Home Office/ASRU guidelines on Animal Testing and Research https://www.gov.uk/guidance/research-and-testing-using-animals, (c) NC3Rs and UFAW, as introduced above, 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 and the NVS will engage in continuous professional development that will ensure best practices in pharmacology, anaesthesia, animal husbandry and welfare. Refinements in best practice will be implemented where appropriate during the lifetime of the project. Sources of information will include, but not limited to, advice from the NVS, I.A.T./LASA journals, and recommendations from the 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. The Universities Federation for Animal Welfare (UFAW) may also be consulted, which is an independent registered charity working with the animal welfare science community worldwide promoting, through scientific and educational activity, improvements in the welfare of animals generally but also includes laboratory animals. Commercial breeders of minipigs also issue training packages and newsletters from which additional refinements in best practice are circulated, for example clicker training of minipigs.

130. Molecular Control of Brain 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

nervous system, vision, development, neurodevelopmental disorders, behaviour

Animal types	Life stages
Zebra fish	adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to identify, characterise and validate candidate genes and pathways that lead to the correct development of the vertebrate nervous system and their roles in the cause of 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 general understanding of the developmental processes that lead to the correct formation of our functional circuitry in the brain is essential to comprehend the complex brain functions such as memory, learning and behaviour and makes it one of the fundamental issues in neuroscience.
But also it is important for processes involving the regrowth of neural connections after injury or disease and for understanding the mechanisms of neurodegenerative diseases. Notably, studying the mechanisms of neural circuit assembly will also help to gain insights

into the bases of a range of mental disorders, such as Schizophrenia, Bipolar Disorder, Depression or Autism Spectrum Disorder (ASD), where links to neurodevelopmental causes, such as the generation of axonal pathways or synapse formation, have been established. In addition, our work on visual perception has a direct relevance to human behaviour, since for example defects in sensory processing have been strongly linked with ASD. We therefore anticipate that our findings may have an impact on clinical applications.

What outputs do you think you will see at the end of this project?

We anticipate new knowledge on several levels:

- identification of genes and mechanisms to form a functional nervous system
- consequences of gene mutations or deletions for structural and functional assembly of the brain and for the behaviour of the animal
- linkage of such genes and mechanisms to the cause of neurodevelopmental disorders

Reporting the new finding will result in publication of peer reviewed articles in international scientific journals.

Who or what will benefit from these outputs, and how?

This project will increase our knowledge how a normal brain develops and how signals coming from the outside (such as information we see with our eyes) are processed to enable us to recognise the world around us. In addition, understanding the assembly of a healthy brain is essential to identify the possible underlying causes of neurological disorders, such as ASD, Bipolar Disorder, Schizophrenia or Depression. Finally, the knowledge created within this program of work will also be crucial to develop therapeutic strategies for brain repair, for example after trauma or disease.

How will you look to maximise the outputs of this work?

As done before, we plan to present our new findings at national and international conferences and publish them in peer reviewed journal articles. Discussions with collaborators and experts in the field will be helpful to optimise and further develop our scientific approaches. We are part of international professional societies where more specialised themes can be discussed.

Consistent with our present strategy, we will continue to organise public engagement events to inform the wider public about our research outcomes.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 43,200 fish

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 use zebrafish. They have a short reproductive cycle and transparent embryos and larvae, therefore enabling easy assessment of their neurodevelopment. There is an approx. 70% sequence homology between the zebrafish and human genome and about 84% of genes known to be associated with human disease have zebrafish counterparts.

The vast majority of our work will be done at embryonic or larval stages, before the animals become protected under the law. Importantly, at that time the nervous system is already mature enough allowing us to study the molecular and cellular mechanisms for its correct development. Similarly, larval zebrafish show a series of well-identified behaviours that can be assessed in relation to human neural disorders.

Typically, what will be done to an animal used in your project?

The vast majority of our work will be done at embryonic or larval stages, before the animals become protected under the law. This includes injections of substances into the embryo (for example to label cellular structures or mis-express genes) or imaging the animal using a microscope.

For animals older than 5 days post-fertilisation, the majority of experiments will include behavioural assessments, which means that free-swimming animals will be observed in their environment under specific conditions (for example fish tanks with light and dark areas, or different social interactions). The duration of these assays can be anywhere between a few minutes up to several hours, depending on the assay.

In addition, some larvae will be undergoing imaging experiments for the structural/functional assessment of their nervous system. Here, non-anaesthetised larvae will be immobilised in a jelly like substance under water during the imaging. Each session can last up to 3 hours, although shorter sessions of about 30 minutes will be the norm. In some cases, up to four separate imaging sessions will be undertaken, always with a recovery period of 24 hours in between.

What are the expected impacts and/or adverse effects for the animals during your project?

No clinical signs are expected in any protocol for our work in this project.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All our work will be at the "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?

Our studies focus on development of the brain and the establishment of the connections between cells. Furthermore, we plan to assess consequences of possible brain wiring defects in behavioural assays. In order to address these questions, the hugely complex neural architecture of the brain must be intact.
Tissue cultures systems do not exist that recapitulate the full functional architecture. Thus, alternatives to using animals are not available, as these processes can only be studied in the living organism.
Therefore, the use of animals for in vivo work is absolutely necessary. Which non-animal alternatives did you consider for use in this project? In vitro assays (cell lines, neuronal cultures)

Why were they not suitable?

Although certain aspects of circuit formation can be studied in vitro (and we will do so wherever possible), the behavioural response of an animal cannot be recapitulated. This also applies to the structural and functional development of neural connectivity in the brain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals according to the nature of our experiments and our long- standing experience working on these subjects. Our work includes a fair number of different genetically altered lines that will be used in combination. Up to 10,000 adults will be used for breeding purposes and the generation of embryos. For our labelling and behavioural experiments, we will use up to 30,000 animals. A large proportion of our project will be done at stages before fish will become protected under the act. In addition, we intend to generate new genome-edited lines to validate disease candidate genes. This includes the screening of potential founders until a line can be established.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Discussions with colleagues working with zebrafish applying similar techniques. Consultation of scientific publications with similar approaches. Interaction with specialist provider of experimental equipment for animal behaviour assessment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Fish will be kept in a state-of-the-art facility with automated feeding mechanisms. Breeding strategies are optimised to keep number of animals low. We will apply small pilot studies for any new substance or technological approach we intend to use for the project. We will use in vitro assays for any question that can be fully or partially addressed before changing into the animal system. We will record multiple measurements (for example responses from different neurons) in the same animals. Where possible, we will also seek to use computational models of neuronal structure and function to extend and complement our in vivo findings.

When generating new models, for example through CRISPR genome editing, appropriate algorithms will be used to maximise predicted on-target and minimise off-target effects. Furthermore, tools designed in this way will then be tested in pilot assays at very early embryonic stages (before animals become protected under the Act), for example to confirm cutting efficiency of guideRNAs. Only tools that have passed this quality control will then be used to generate mutant fish, in order to minimise unsuccessful attempts and therefore reduce 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.

We will use zebrafish to conduct our research. There is an approx. 70% sequence homology between the zebrafish and human genome and about 84% of genes known to be associated with human disease have zebrafish counterparts. Moreover, the zebrafish brain shows similar neuroanatomy to the human brain and uses conserved neurotransmitter pathways. At the same time, there are several advantages using this system: It allows us to follow the structural and functional development of the nervous system in the embryo and larva, completely independent of the mother. Zebrafish are optically transparent, therefore in vivo imaging is possible without the need for any surgery or other procedures. Our methods will include the generation or breeding of genetically altered zebrafish lines. A number of fish will be used for non-invasive live imaging purposes, which is not harmful to the animals.
Furthermore, some of the methods include the behavioural assessment in free-swimming animals, which simply requires the observation of animals. This therefore does not cause any pain, suffering or lasting harm. Finally, the majority of our experiments will be done at embryonic or larval stages before the animals become protected under the act.

Why can’t you use animals that are less sentient?

The majority of our experiments will be done at embryonic or larval stages before the animals become protected under the Act. All the protocols are under the "Mild" severity level. As outlined in an article by the NC3Rs (Brennan, NC3Rs website 2014), zebrafish make excellent research models compared to other vertebrate systems, due to the advantages outline above. We therefore have chosen the lowest sentience currently

possible for this type of work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We previously generated a new completely transparent zebrafish line to enable unobstructed imaging without the need for applying pigmentation inhibitors (drugs) that have a negative effect on animal health. We will continue to optimise approaches and technologies that will improve welfare of animals. All our protocols are on the "mild" severity level.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practices are followed according to the different method, for example:

Zebrafish husbandry (centrally optimised through our Biological Services Unit)

- Alestrom et al (2020): Zebrafish: Housing and husbandry recommendations, Laboratory Animals 54: 213–224.
- Reed & Jennings (2011) Guidance on the housing and care of Zebrafish (RSPCA Publication).

Imaging

- Abu-Siniyeh et al (2020) Highlights on selected microscopy techniques to study zebrafish developmental biology. Laboratory Animal Research 36: 12.
- Antinucci, P., Hindges, R. A crystal-clear zebrafish for in vivo imaging. Sci Rep 6, 29490 (2016). https://doi.org/10.1038/srep29490.

Optogenetics

- Antinucci et al (2020) A calibrated optogenetic toolbox of stable zebrafish opsin lines. eLife 2020;9:e54937

Behaviour

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How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular meetings withing the zebrafish community, for example through the membership and interactions in societies (International Zebrafish Society, Zebrafish Disease Models

Society) and scientific congresses. Close interaction with the NC3Rs through Zebrafish workgroup and events/publications.

131. Biocompatibility (tests for local effects after implantation) of medical devices and materials

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

Biocompatibility, Hard/soft Tissue Response, Cell Response

Animal types	Life stages
Rats	adult
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?

To conduct safety evaluation of novel products and/or materials in order to demonstrate/ensure that there are no adverse effect when the product/material is used in patients. These materials may be; but not limited to, polymeric (resorbable or non-resorbable, or a combination of both) metallic or ceramic. Work carried out under this licence will feed into programmes of work authorised by other Project Licences, allowing evaluation of efficacy for the suitability in the use of medical devices.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
There is a proven track record of innovative research leading to new products/techniques in the fields of Orthopaedics and Regenerative Medicine. An integral part of this research is the use of in vivo models for both safety and efficacy testing. Biocompatibility testing is

an important part of the development of materials and devices that will be implanted into or will interact with hard/soft tissue. Specific reference will be made to the requirements of BS EN ISO 10993 part 6 [1] during biocompatibility testing using in vivo models.

What outputs do you think you will see at the end of this project?

This licence will facilitate the development of new products and allow clinically relevant improvements to existing treatments in the field of hard and soft tissue repair. The purpose of the development of these new products and materials is to help patients return to normal levels of activity in as short a time period as possible.

Who or what will benefit from these outputs, and how?

According to the National Joint Registry (2020) in England and Wales 160,000 total hip and knee replacement procedures are performed each year. The U.S Agency for Healthcare Research and Quality (2020) state more than 450,000 total hip replacements are performed each year in the United States. Worldwide, 1 million young athletes visit hospital for sports related injuries. One of the most common injuries is that of the anterior cruciate ligament. According to the American Orthopedic Society for Sports Medicine(2018), around 150,000 ACL injuries occur in the United States every year, costing 500 million USD in U.S. health-care costs each year.

SHORT-TERM BENEFITS (0-3yrs): These animal models will help to facilitate the development of innovative products, which will improve patient outcomes including, ability to return to a day-to-day life much quicker and experience less pain. The project will attempt to aid in the development of novel products for bone/soft tissue repair and bone ingrowth, which will help advance the frontiers of science in this area for the benefit of clinicians appraising preclinical trauma, bone ingrowth and soft tissue repair studies.

LONG-TERM BENEFITS (5-10 years): The project will aid in the provision of long term benefits for patients undergoing limb reconstruction, joint replacement or sports injury. The ability to develop additional/new therapies to address such issues will improve the overall management of these patients, allowing the economic benefits of fewer days lost at work, fewer hospital days and reduced care costs.

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 or product claim sheets, (b) pre-clinical regulatory support data, (c) conference abstracts and posters, promoting knowledge transfer, (d) unsuccessful approaches are captured within a Master Safety Assessment, which is shared with regulatory bodies and internally.

Product development may also involve the support of either academics or clinicians, which will assist with knowledge transfer. The outputs from these studies will be archived and may be used by the research team initiating the line of work, as well as other research teams, as historical data for internal learning. Best practice may also be disseminated via, for example, the I.A.T. animal welfare discussion group, upon request.

Species and numbers of animals expected to be used

- Rats: 100

- Rabbits: 100
- Sheep: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

RATS: Rats have been selected as a small animal model given their long history of use in literature for this model. Adult rats are the preferred choice for this licence due to (a) the ease of supply from registered breeders (b) the size and number of implants and (c) intended duration of the test in relation to the expected life expectancy.

RABBITS: Rabbit have been selected as a small animal model as they are a useful intermediate prior to progressing to the larger animal model, which may be a better representation of the clinical setting. Adult rabbits are the preferred choice for this licence due to (a) the ease of supply from registered breeders (b) the size and number of implants and (c) intended duration of the test in relation to the expected life expectancy.

SHEEP: Sheep have been selected as a large animal experimental model given that their anatomical size and skeletal dimensions is similar to humans. The use of sheep provides an ideal test bed for testing implants of various sizes and composition in the same animal. Skeletally mature sheep are preferred over juvenile and aged animals in this licence for the following reasons; (a) availability from the open market, (b) our current implants and surgical instrumentation also tailored towards the bone of skeletally mature animals which is comparable to human, (c) bone repair and remodeling processes are dissimilar between juvenile and skeletally mature animals and so juvenile animals are not considered to be sufficiently representative of the clinical conditions under study, (d) from past experiences, aged sheep are more susceptible to succumbing to complications arising from the surgical procedure and the effects of the general anesthetic. Therefore, for this reason young adults are more suitable.

Typically, what will be done to an animal used in your project?

Rat or Rabbit Subcutaneous/Intramuscular Implantation: Animals will enter the facility and undergo a period of acclimatisation, lasting a minimum of one week but is likely to be for a period of three to four weeks. Animals will be group housed. Rats will be grouped as per transport groups. Rabbits will be grouped in conjunction with identification numbers given by the supplier, group housing will be encouraged but if there are signs of fighting then certain individuals may be singly housed, although sight and smell will be possible. During the acclimatisation (and post-surgery) period animals will receive environmental enrichment (hay, balls, toys and chews etc.) in addition to regular staff contact. Surgically, under general anesthesia with recovery, a test material will be implanted either under the skin, or into a muscle bed site of a suitable size of a rat/rabbit to accommodate the implant specimens e.g. gluteus maximus. The estimated time of the surgical procedure is approximately 1 hr. Following surgery recovered animals will be returned to their original group housing and will be carefully monitored by qualified animal husbandry staff until the live phase of the study is completed up to 26 weeks later. At the end of the study, the animals will be humanely euthanized. Occasionally, blood sampling and radiographic imaging; with anaesthesia, may be required.

Sheep Bone Implantation: Animals will enter the facility and undergo a period of acclimatisation, lasting a minimum of one week but is likely to be for a period of three to four weeks. Animals will be group housed. Surgically, under general anesthesia with recovery, cylindrical defects (maximum size of 10mm diameter x 20mm depth) will be created in the proximal tibia and/or distal femur of skeletally mature sheep. These defects will be filled with either test materials, industry standard controls or left as empty defects. The defects may be created in both hind limbs; up to four defects will be created per animal. The estimated time of the surgical procedure is approximately 1.5 hrs. Sheep will be singly housed until fully recovered, group housing will be encouraged as soon after surgery as possible to minimise stress. 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 3 years later. At the end of the study, the animals will be humanely euthanized. Occasionally, bloody sampling, radiographic imaging; with anaesthesia, and administration of bone markers may be required.

What are the expected impacts and/or adverse effects for the animals during your project?

Subcutaneous/Intramuscular Implantation in Rats and/or Rabbits: On arrival the animals will be subjected to a new environment and its associated stress (in addition to transportation stress), hence the acclimatisation period. The project will involve testing the local effects after implantation of a test material. The test material is implanted (under general anaesthetic with recovery) either under the skin, or into a muscle bed site of suitable size to accommodate the implant specimens. Post surgery some evidence of tissue reaction may be seen, however, very little due to the pre-testing of the material for cytotoxic and irritant effects. Surgery may also lead to some degree of discomfort, although this will be reduced by the use of a minimally invasive surgical technique. Any discomfort will be minimised with the use of 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 infection) will be humanely euthanised.

Bone Implantation in Sheep: On arrival the animals will be subjected to a new environment and its associated stress (in addition to transportation stress), hence the acclimatisation period. The project will involve the creation of a bone defect, which may lead to some degree of discomfort following surgery, although this will be reduced by the use of a minimally invasive surgical technique. Post surgery some evidence of tissue reaction may be seen, however, very little due to the pre-testing of the material for cytotoxic and irritant effects. Any discomfort will be minimised with the use of 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)?

Subcutaneous/Intramuscular Implantation Rats and/or Rabbits: It is expected that greater than 97% of animals will experience moderate harm under procedure. The remaining 3%

or less of animals will be classified as non-recovery by dying whilst still under controlled anaesthesia due to either adverse reaction to the anaesthetic itself or being euthanised due to irreparable surgical complications.

Bone Implantation in Sheep: It is expected that greater than 98% of animals will experience moderate harm in work under this protocol. The remaining 2% or less of animals will be classified as non- recovery by dying whilst still under controlled anaesthesia due to either adverse reaction to the anaesthetic itself or being euthanised due to irreparable surgical complications.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals will only be used where there are no alternatives to the use of animals in order to answer the questions that the project requires. In-vivo models will be required when in-vitro systems cannot provide a reproducible approximation of the real-life in-vivo or clinical setting, e.g. the biocompatibility and degradation properties of implant materials. Soft/hard tissue responses to a foreign body; for example inflammation, is a complex process involving a multiple cascade of repair responses that cannot currently be reproduced in an in-vitro setting.

It is a legal requirement to conduct biocompatibility testing on new products/materials for medical devices on live animals.

Which non-animal alternatives did you consider for use in this project?

In-vitro cell culture models will be used to establish safety of any technology prior to any in-vivo experimentation, these preliminary tests will be used to screen out novel technologies that lack supportive data.

Consideration of in-vitro cellular repair mechanisms and inflammation was made, however currently, there are no suitable models to evaluate tissue response without the use of animals.

Why were they not suitable?

In order to provide a sound basis for making determinations about reasonable safety and efficacy, animal models will be required to provide accurate information about how a medical intervention will perform in a human clinical trial, however, as previously described multiple cellular interactions in repair and repair cascade can not be replicated in-vitro.

Furthermore, regulatory authorities such as the FDA and MHRA will require evidence of in-vivo safety and efficacy data prior to clinical evaluation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

It is estimated that the clinical needs that this project licence is required to address will result in studies utilising approximately 20 animals per protocol per year. This is based on 1 study per protocol per year. Minimum animal numbers are prescribed in standards (ISO 10996 part 6, 'Tests for local effects following implantation') and are followed, however, animal numbers maybe reduced by implanting multiple test samples in one animal.

Multiple strategies will be adopted 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.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

STATISTICS: The numbers have been estimated based on anticipated numbers of studies required over the 5 year duration of the project and the group sizes stipulated by the International Standard. When necessary, statisticians will be consulted in the planning stage of the in-vivo studies, to determine the minimum number of animals required 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.

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.

MULTIPLE TEST SITE/CONTROLS - The standard allows for controls and multiple test sites in a single animal to aid in the reduction of total animals used per study.

APPROPRIATE USE OF ENDPOINTS - TISSUE SHARING: Where possible, harvested tissues will be recycled for multiple testing, e.g. blood draws, biopsies and histology .

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: To establish safety of any product/material prior to any in-vivo experimentation

QUALITY ANIMALS: The loss of animals can be minimized by providing good pre/post-operative care, avoiding unintended breeding, and planning ahead so that the appropriate number of animals needed for the studies are ordered and/or bred.

PILOT WELFARE STUDIES: Previous studies, studies reported in literature or pilot studies can be used to estimate variability and evaluate procedures and effects.

APPROPRIATE USE OF ENDPOINTS - TISSUE SHARING: Where possible, harvested tissues will be recycled for multiple testing, e.g. blood draws, radiographs and histology.

LOCAL ETHICS (APPROPRIATE EXPERIMENTAL DESIGN): Our 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.

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.

These animal models are prescribed within ISO:10993:part 6. The standards are internationally agreed and accepted by experts and are the models that obtain the required information causing the least possible suffering to the animal. Techniques and husbandry have been refined over the years within our own establishment and in the wider animal science community to generate widely accepted best practice.

There has been accrued a great deal of experience with these animal species from previous licenses, which in turn has led to refinements in surgical technique, analgesic regimes and post-surgical care

Why can’t you use animals that are less sentient?

Live mammalian vertebrates are required that closely mimic the bone repair pathways and human skeletal system as much as possible to ensure that any data generated can be translated to the clinical situation.

The protocols assigned to the less sentient species (rat and rabbits) are generally intended to be used for the initial/earlier stage assessment of technologies. However, as the technologies progress through the product design control matrix, there will be a requirement to assess their safety and efficacy in animal species whose anatomy and physiology more closely represent the intended use in humans.
These animals (sheep) tend to be of higher sentience.

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/handling of animals and group housing to habituate animals to study procedures to minimise distress, (e) implementation of housing, e.g. micro- and macro-environment, (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 stress/pain and (i) pre/post-operative care/recovery and (j) pain management (anesthesia, analgesia, drug pumps).
SOPs will also be regularly updated and documented within our Quality Management System. Staff training will also be made available through attending courses and conferences and integrating with key opinion leaders. We have a great deal of experience with all of the protocols described in this licence. This experience has led to refinements in surgical technique, analgesic regimes and post- surgical care.

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 animal required to obtain the desired research objectives. Best practice on animal care and husbandry will also be achieved through several sources including (a) Animal Welfare 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?

I will stay informed on these topics by regular reviewing of literature, attendance of conferences, membership of organisations; for example LASA, IAT, NC3R's, RSPCA, allowing me access to relevant, current and best practice materials and methods. I am part of an animal welfare group which permits the sharing of knowledge between industry professionals.

SOPs will also be regularly updated and documented within our Quality Management System. Staff training will also be made available through attending courses and conferences and integrating with key opinion leaders.

Home Office

132. Tumour models for the characterisation and therapy of advanced 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, resistance, angiogenesis, orthotopic

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We are aiming to improve our understanding of how cancers spread round the body and develop resistance to drug treatment. Our aim is to develop clinically relevant animal models of advanced stage human cancers. Cancers at this stage are difficult to treat and have very poor survival rates. We wish to better understand the cellular and molecular basis of how cancers grow, spread and develop resistance to treatment. We can then apply this knowledge to the discovery and preclinical development of novel agents emerging from the groups work.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Survival rates for many cancers have improved significantly over recent decades, but the disease remains extremely difficult to treat once it has spread around the body. Cancer at

this stage can only be treated with systemic therapies that affect the entire body, and the disease is able to adapt, evolve and so becomes drug resistant.

Traditionally, cancers that have spread have been treated with very toxic chemotherapy drugs, with attendant harmful side effects on normal self-renewing tissues. Increasingly efforts are being made to develop new forms of “precision medicine", which target specific genetic changes in each cancer. To do this we must be able to identify these changes in genes of interest and see how the changes affect marker molecules within the cells. It is important to understand how cancers can develop resistance to these new drugs. And to open up new avenues of treatment, we need to identify the molecules that encourage and facilitate the spread and growth of the cancer away from the primary tumour site.

What outputs do you think you will see at the end of this project?

We expect to make advances in the understanding of how cancers grow, develop blood supplies, spread and develop resistance to therapy. We expect to increase our understanding of the use of markers in blood or tumour samples for patient selection and to check that therapies are producing the desired effect. We also expect to make progress in our understanding of how the immune system interacts with cancer.

We aim to support the future discovery of precision medicines that target invasive, spreading, drug- resistant cancer while sparing normal proliferating cells. A greater understanding of how new blood vessels develop to support cancer growth, what causes cancer to spread and what helps new tumours to establish in secondary sites around the body will help the research team identify new treatment targets and tackle the unmet clinical needs of cancer patients.

We publish extensively, including many papers on refined methods both using cells in the laboratory and tumour models in animals. Our findings will continue to be reported in academic publications and at national and international conferences. Validated models will be made available to the research community.

Who or what will benefit from these outputs, and how?

Basic science – The novel information acquired and the model systems developed from this proposal will have a direct benefit for both national and international academic institutions that are carrying out research into cancer

Clinical translation : Our animal models will provide important information to clinicians about how cancers become resistant to treatment. Understanding drug resistance will help clinicians in making decisions about how to treat patients more effectively and in the design of new treatment strategies. The development and testing of clinically relevant tumour models for treatment will improve our confidence that agents going forward to clinical trial can successfully reach and shrink tumours where they naturally occur in the body, including in challenging sites that are difficult for drugs to reach, such as bone or brain. The detection and use of chemical markers in patient blood or biopsy samples to select the most appropriate patients for trial and then to monitor response to treatment will be invaluable to ensure we select the patients for clinical trials who are most likely to benefit from a treatment and can assess treatment response as early as possible.

Patient benefit: This work should directly produce new treatment strategies for the hardest to treat cancers that currently have few treatment options available. We hope to

increase the overall survival and quality of life of these patients

How will you look to maximise the outputs of this work?

How information will be disseminated: All information gathered from our studies will be shared and discussed with teams within our unit to maximise the benefit to all our research programmes. We will disseminate findings externally through a wide range of channels including presentations and posters at relevant conferences, organisation of workshops and publications in relevant journals. In addition we will also announce breakthroughs and updates through social media channels such as Twitter, the institutional website and where appropriate through the news media.

Species and numbers of animals expected to be used

- Mice: 7060

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 model species for this investigation as they are the lowest animals in the evolutionary tree in which suitable models of cancer treatment can be carried out. The project requires genetically modified mice strains that are immunocompromised to allow the growth of human cell lines and prevent rejection by the mouse immune system.

All experiments will be conducted in adult mice.

Typically, what will be done to an animal used in your project?

1. Mice will receive an injection of tumour cells to induce tumour formation, usually in the correct physiological location e.g. liver tumour into liver etc. which may be via a surgical procedure or using ultrasound guidance which results in less tissue damage than surgery.

2. Animals will be assessed for tumour initiation either by observation and gently feeling for surface tumours or by imaging using non-invasive techniques to monitor tumour development. Once tumours have reached treatment size (typically within 2-8 weeks) the animals will be randomised into groups for treatment.

3. Tumour-bearing animals will be given either single or more usually multiple doses of therapeutic agents dependant upon the properties of the agent ie. once or twice daily or the route of administration e.g. intravenous or by mouth. Sometimes more than one therapeutic agent will be given (combination therapy) and sometimes therapeutic agents are given alongside other treatment methods e.g. radiotherapy or car-T cells.

4. Not all animals will be treated. Some will be used for tumour characterisation studies. Some will be used to assess the genetic changes within the cancer that have led to its formation of spread around the body. Others may be used to monitor biomarkers of

cancer within the body.

5. Animals will be monitored by either simply measuring the size of surface tumours or via imaging non-invasively to follow treatment outcomes such as changes in tumour size and tumour cell death. The typical duration of experiments will be 1 to 3 months depending on the growth rate of the tumour.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will undergo tumour development and tumour treatment and as a result of this may experience weight loss or a general loss of condition. Animals will be checked daily and weighed up to 2 to 3 times a week . The body condition and behaviour of animals will also be assessed at these times.

Surgical procedures and cell injections can cause internal bleeding or small blood clots that can lead to blocked blood vessels. Animals are assessed rigorously during procedures and directly afterwards upon recovery, and any animal showing evidence of bleeding or blocked blood vessels will be humanely culled immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities are mild to moderate. Some 50% of animals will experience only mild effects with few side effects and with tumour regressions due to therapy. Some 50% will have moderate effects with more loss of condition due to treatment (similar to that seen in humans) and having tumours of larger size e.g. control animals who receive placebo 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?

Human cancers develop within specific tissues in the body, each providing a unique micro-environment. All the cells within a tissue can contribute to tumour formation and growth-not just the cancer cells we implant but the cells that make up the surrounding 'normal' tissue. These complex conditions and interactions between cells cannot be adequately modelled in the laboratory without the use of animals. What is more the spread of cancer from its primary site to distant secondary sites (the major cause of treatment failure) is a phenomenon only fully seen in whole animals. Since cells need to access the circulation in order to move to and survive at secondary sites this research can only be done in whole live animals. Similarly, the effects of drugs must be tested in animals to determine that

adequate drug levels are achieved in tumour tissues, that adverse effects on normal tissues are minimised and that efficacy tracks with measurable chemical markers in the blood or tumour – i.e. evidence that the compound reaches its target and selectively inhibits it. With metastatic disease, it is especially important to determine that all sites of disease can be accessed and the drug target inhibited. Some anticancer agents can also inhibit the growth of new blood vessels, which cancers need to establish and thrive, so it is essential to monitor this too. Effective vascular targeting may demand different dosing schedules and the monitoring of specific markers in the blood and tumour.

Which non-animal alternatives did you consider for use in this project?

We have carried out extensive studies in the laboratory without the use of animals to mimic as many of the basic cellular processes as possible. We have developed a battery of high-throughput 2D and 3D assays of tumour cell proliferation, migration, invasion, and enzyme activity (processes involved in metastasis) and also use endothelial cells (which make blood vessels) in multiple assays of new blood vessel development.

Why were they not suitable?

None of these assays adequately predicts the complexity of in vivo responses in whole live animals. The advanced studies in this licence are only carried out on agents that have been triaged through PK/PD analyses and simple tumour models and show promising activity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 we will use based largely on previous experiments and the literature. We have extensive prior experience of using these models and therefore have a good idea of how many mice we require to achieve our aims. Based on our previous data we will use the minimum number of animals required to give a statistically meaningful result. We will consult with our bioinformatics team for advice on experimental design to keep numbers used as low as possible and choose the most appropriate statistical methods to analyse our results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All animal studies are designed with assistance from the NC3Rs Experimental Design Assistant and using ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. We will consult with a statistician with regards to the experimental design to minimise the number of animals used whilst ensuring meaningful data can be collected.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For tumour studies, we aim to use the minimum number of mice per group that will be informative. The animal numbers required to obtain significant results are dependent on the particular tumour model.
The most important characteristic of a model is the reproducibility and thus predictability of tumour development. We use inbred animals that show very little variability and all consistently show the same growth characteristics for a given tumour. Our use of immunocompromised animals ensures we do not experience rejection of tumours by the host immune system. Initial checks on whether a particular tumour grows and experiments to characterise the tumour – i.e.' pilot studies' to find the most reliable cell line or genetically manipulated variant clones - will already have been done on another of our licences. As far as possible, internal tumour development is monitored by non-invasive imaging, especially bioluminescence studies for metastasis models. This approach enables us to monitor how cancers grow, develop and spread in a single mouse over time. This greatly reduces the number of mice needed for each experiment as we get data from the same animal many times. We have also pioneered the use of 'microcapillary bleeds' (~10µL blood samples) together with highly sensitive mass spectrometric methods to assess circulating drug levels during therapy and to detect the chemical markers of drug action - This once again reduces the need for large groups of animals for repeated sampling as we can take many very small samples from the same mouse over 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.

Mice are the lowest species that are appropriate for in vivo drug development studies and are widely used for this purpose. The mouse is considered to be the lowest mammal species we can use that gives correlation to man. Most of our work is carried out using well-characterised human tumours, grown in the correct anatomical site in mice that are bred to be immunodeficient (nu/nu, SCID or NSG mice) to avoid tissue rejection. This enables us to study human cancers in the correct tissue microenvironment (albeit in a mouse host). The animals are maintained in individually ventilated cages using sterile food and bedding and all procedures are carried out in laminar flow cabinets to avoid infections.

The animal models we have chosen are well characterised and well documented to produce reliable results while only having moderate effects on the mice. We use well-established methodologies that we know have little or no adverse effects, by themselves, to activate or inactivate specific genes. We will then monitor mice for signs of tumour development, typically including weight loss, inactivity or sometimes other specific characteristics of a particular tumour type all the while minimising pain, suffering and distress.

By using cutting-edge preclinical imaging technologies, such as ultrasound and magnetic resonance imaging, we will ensure that injection of tumour cells into the animals is

performed with the highest possible precision. This will increase success rates and reduce the impact of procedures on animal wellbeing (for example by reducing the need for surgery). Imaging also allows for monitoring of tumour growth and ensures we can precisely define the end points of experiments so we can reduce the impact on the mice.

Suffering will be minimised by keeping tumour burdens within tolerable and acceptable limits detailed in each protocol as appropriate.

Compounds to be evaluated will have been triaged for potency, stability and tolerability in other projects, and are generally of low toxicity (e.g. agents targeted to molecules selectively overexpressed or mutated in human cancers). None of the protocols is categorised as ‘substantial’ severity.

Why can’t you use animals that are less sentient?

Mice are the lowest species that are appropriate for in vivo drug development studies and are widely used for this purpose. For our studies of late-stage cancer we need to use adult mice as this is more physiologically relevant to man. In addition, we will be studying how cancer grows and responds to treatment over a period of time that could be as long as six months, so the use of young or terminally anaesthetised mice would be impractical.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our aim is to create new therapeutic regimes for the treatment of advanced human cancer. We will not subject any animal to tumour implantation and treatment until we are sure we have enough invitro evidence that his could be an effective treatment strategy which it would be possible to transfer to patients in the clinic.

Animals undergoing surgical procedures will receive anaesthesia and pain relief. After surgery, the animals will be intensely monitored until they have recovered from the anaesthesia. If there are no clinical signs and the wound shows no swelling or bleeding, the animals will be monitored 2 to 3 times a week. We are continually developing technologies to refine our experiments and to minimise suffering of our research animals. Non-surgical ultrasound-guided injection will be performed in some groups of animals to deliver tumour cells with accuracy and less tissue damage than surgery. This reduces any risk of infection, greatly increases the rate of recovery and limits pain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidance given in the NC3Rs ‘Resource Hub’ (https://nc3rs.org.uk/resource-hubs) for example on blood sampling (https://www.nc3rs.org.uk/blood-sampling-mouse). We will also refer to the National Cancer Research Institute guidelines on using animals in cancer research published by Workman et al 2010 (British Journal of Cancer 102, 1555 – 1577).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with the latest developments on refining animal research methods via the NC3Rs website (https://www.nc3rs.org.uk). Animal house staff will ensure that any

advances are fully implemented throughout the facility.

133. Inter-connected studies of prion 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

Prion disease, Transgenic animals, Infectious materials, Antibody production, Behavioural studies

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo, aged
Hamsters	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 Unit’s mission is inherently long term and challenging: essentially to understand the molecular basis of mammalian prion propagation and neurotoxicity, and to deliver the means for the early diagnosis and effective treatment of human infection and disease. As laboratory rodents are naturally susceptible to prion infection, and our transgenic mice faithfully propagate human prion strains and fully recapitulate human pathology, there are reasonable grounds for optimism of successful translation of preclinical therapeutic results to 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 purpose of this work is to embark on basic research likely to yield results that can be

translated into more reliable prion disease diagnosis and ultimately treatment in patients.

What outputs do you think you will see at the end of this project?

Short-term benefits are expected to include a better understanding of prion biology, a vital area of public health concern. Such knowledge is essential in our search for effective therapeutics for these invariably fatal diseases for which there are at present no treatment options. Prion and other neurodegenerative diseases share the commonality of protein misfolding disease mechanism and it is therefore likely that therapies found for prion diseases will have wider application for treating other neurodegenerative diseases. A better understanding of prion strains is critical to inform on fundamental aspects of prion biology and their wider relevance.

Who or what will benefit from these outputs, and how?

This research is critical to understanding the molecular mechanism of prion replication and strain determination, and will have direct translational benefits for both rational therapeutics (being investigated under a separate project licence) and prion disease diagnosis. Ultimately, patients will benefit from the outputs of this research.

How will you look to maximise the outputs of this work?

The research of the Unit is strategically inter-connected, thus maximising a range of skills and expertise to ensure that the most important questions in prion biology are being tackled from many different angles. In addition to the internal close working relationships, we also work with several external collaborators on specific aspects of our research programmes. The Unit provides an internationally recognised centre of expertise in post-graduate training, prion biosecurity and specialist technical and advisory expertise to academic centres, industry, WHO and Government at a national, EU and international level. Any new knowledge acquired through the conduct of this project will facilitate the dissemination of knowledge and experience nationally and more widely.

Species and numbers of animals expected to be used

- Mice: 127,800
- Hamsters: 1,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
Mice will be used as their genetics, physiology and neuroanatomy are very well characterised and their breeding time and lifespan allow experiments to be performed on a manageable and affordable timescale. Their similarity to humans in terms of genetics and neuroanatomy, and their genetic tractability, which allows for the generation of genetic models of human diseases, has led to the generation of many successful models of neurodegenerative disease and the identification of potential therapeutic avenues. For these reasons, the mouse is our model of choice for gaining new insight into neurodegenerative disease mechanisms that are likely to be relevant to humans. Different

stages of the mouse (embryos, juvenile, adult) will be used as appropriate for each protocol. For a limited number of studies Hamsters will be used to study specific prion strains that may not propagate in mice.

Typically, what will be done to an animal used in your project?

This interconnected project on prion diseases has 13 protocols in total, and procedures will vary depending on the specifics of the protocol. Protocols 1-4 for the generation and characterisation of new transgenic mice will involve hormone injections to egg-donor mice, surgical procedures in vasectomised males and oviduct or uterine transfer in recipient mice. Protocols 5 and 6 will be for the breeding and maintenance of GM and wild type mice. Protocols 7 and 8 are for the ageing of mice for the purposes of sample collection, and the generation of primary cell cultures respectively. In protocols 9-13 some animals will be inoculated with prions or other substances under general anaesthesia in order to study prion transmission properties, phenotypic assessment and passive immunisation for the purposes of antibody production.

What are the expected impacts and/or adverse effects for the animals during your project?

Female mice for superovulation will be of an appropriate size, in particular if they are to be mated. Over-vigorous males will be replaced.

Animals undergoing surgical procedures carried out aseptically under general anaesthesia may rarely develop post-operative complications. Such animals will be humanely killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery.

Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Any animals that fail to do so or exhibit signs of pain, distress or of significant ill-health 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) will be humanely killed by a Schedule 1 method.

Occasionally, the clinical course of scrapie (an alternative terminology for prion disease in animals, but the two terminologies may be used interchangeably from here on) may proceed unexpectedly fast and more severe signs may be noted. These occasional severe signs will not exceed 2% of the total number of animals to be inoculated with prions.

In all other cases, any animal showing any deviation from normal health or wellbeing will be immediately humanely killed by a Schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of mice (84%) are expected to have a severity of sub-threshold, about 8%

mild and 6% used in prion transmission experiments will have moderate threshold. It is anticipated that the severe threshold may be reached in up to 2% of the animals used, but animals will not be deliberately allowed to progress to this stage. A small number of hamsters used for prion transmissions will have a moderate threshold.

What will happen to animals at the end of this project?

- **Killed**

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand the fundamentals of prion disease, develop therapeutics (under a separate project licence), improved decontamination methods and diagnostic tools, effective and appropriate experimental models are essential. While we have cell culture facilities for studying rodent prions in the test tube, there are at present no cell lines for studying human prions efficiently. We have parallel projects aimed at developing cell lines for studying human prions, and these have begun to yield promising results. We now have cell lines that can propagate only certain human prion strains. When fully developed and refined, the use of these new cell lines will allow us to perform some of our prion infectivity studies in vitro. However, until this test tube work has been successful in the near future and extended to many more human prion strains than has been possible thus far, key prion disease parameters such as clinical duration and features, behavioural changes, brain damage and the spread of the abnormal protein within the body leading to fatal brain damage, can only be studied in an animal.

Which non-animal alternatives did you consider for use in this project?

Cell culture and automated scrapie cell assay developed in-house will be used for some of our studies. However the use of the whole animal is unavoidable for the study of some key prion disease parameters such as clinical duration and features, behavioural changes, patterns of brain damage and the spread of the abnormal protein within the body leading to fatal brain damage.

Why were they not suitable?

Cell culture facilities are suitable for studying rodent prions in the test tube but not every biological parameter can be studied in culture. Additionally, similar cell lines for propagating human prions are not yet available, though we have made progress in our quest for such a facility. Even when human prion susceptible cell lines are developed, some parameters can only be studied in the whole animal. Fly models of prion disease are used by some researchers, but this model cannot faithfully recapitulate the distinguishing disease phenotypes of human prion diseases. For instance, the neuropathological hallmark of vCJD, that of abundant florid plaques in the brain, has to-date only been demonstrated in transgenic mice that are specifically homozygous for PRNP (human prion protein gene) polymorphic residue 129.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

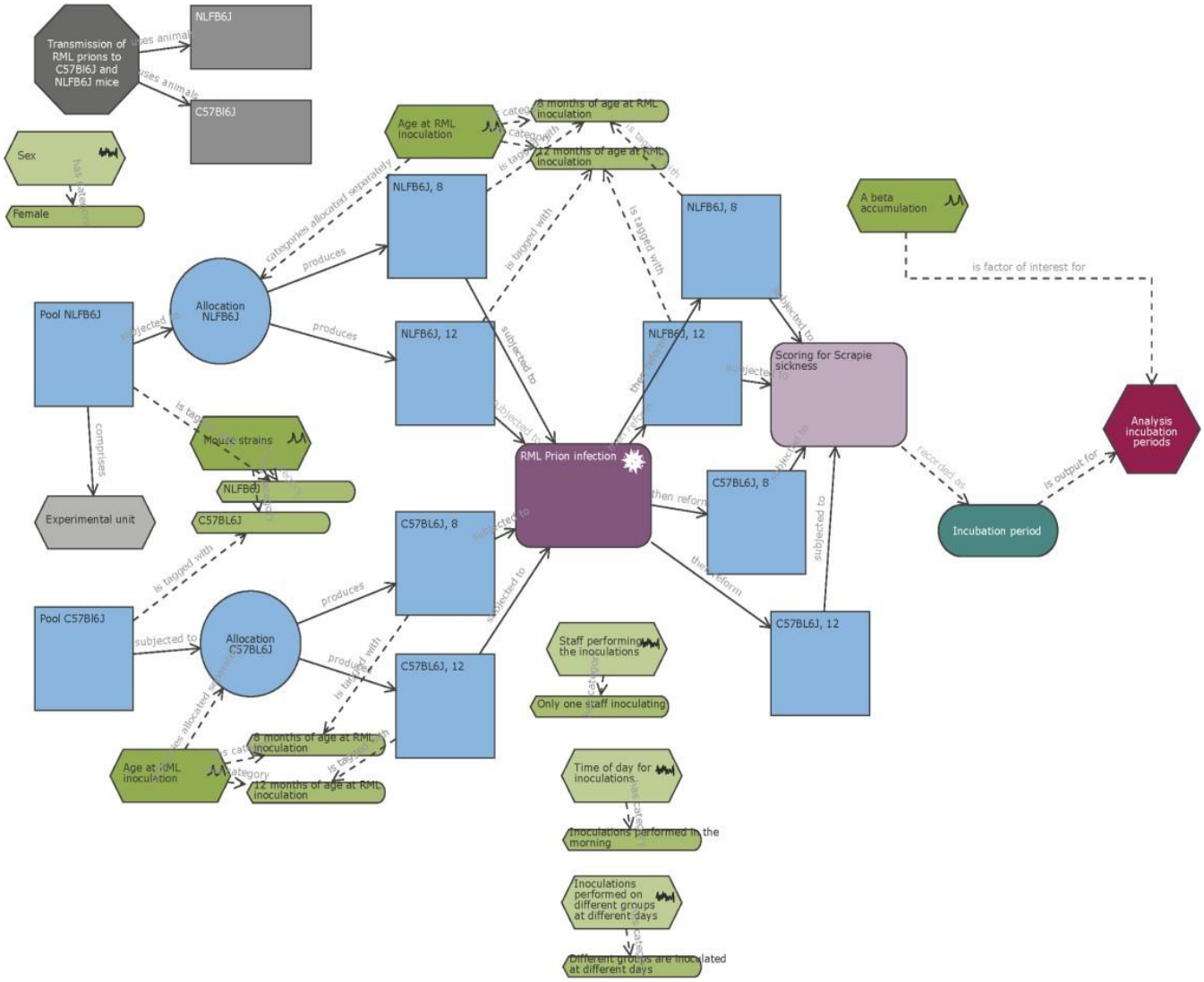
How have you estimated the numbers of animals you will use?

For comparison of prion incubation times and strain-specific patterns of neuropathology, groups of mice of approximately 10-20 will be used based on what is known about how particular prion inocula behave in specific mouse strains. Large scale amplification of particular prion strains for biochemical studies involves larger numbers of mice (up to 100) that are required in order to isolate purified prions in the microgram quantities required for structural analyses. These transmissions involve inoculation of high titres of infectivity in which the properties of the prion/host combinations are known in detail, meaning that the mice are humanely killed at the earliest clinical signs of disease thereby reducing animal suffering. For transmissions using novel mouse or human prion strains, the transmission properties are unknown and the incubation time may exceed the life-expectancy of the mouse. In these instances experimental output is often qualitative therefore group sizes of 10-20 will be used as this allows for sufficient tissue to be available at the end of the experiment.

We use our scrapie cell assay extensively and this has enabled us to reduce the number of mice we would otherwise use in our studies. Use of the newly developed cell lines that have limited susceptibility to some human prion strains will lead to further reduction in the use of mice. For all our research we will ensure that the smallest number of animals will be used consistent with achieving a clear experimental result. We also regularly check our mouse colonies to ensure efficient colony management.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Some of our prion transmission experiments are associated with short incubation periods and low rates of death due to intercurrent illnesses. For such experiments NC3Rs' Experimental Design Assistant (EDA) will be used during the design phase, as exemplified with a typical experiment in the diagram below. The full EDA report is 4 pages long, and can be made available if requested.



However, most of the experiments in this project do not yield quantitative data and are therefore not amenable to NC3Rs' Experimental Design Assistant or other quantitative methods for estimating the number of animals to use in experiments. Notably, Protocols 1-4 encompassing the generation and characterisation of transgenic mice do not yield quantitative data, and so estimation of numbers of animals is based on our 25-year experience. Typically one can estimate that 100 single-celled embryos for microinjection can be obtained from 5 superovulated female mice (Protocol 1) on the assumption that each superovulated female will yield 20 embryos. However, there are so many variables involved in this process that the reality is not easily predictable. For instance, only 3 out of 5 of the female mice superovulated may mate successfully, thus yielding fewer eggs than expected. In addition, different mouse strains have different egg yields, not all recovered embryos are suitable for microinjection, and survival of embryos following the injection process is also variable. The only reliable method for estimating the number of mice needed for Protocols 1-4 is our prior knowledge of the strain of mice, and making the best of the number of embryos on the day.

For prion transmissions that cross species barriers or involve novel transgenic lines or human prion isolates, the incubation time may be unknown and/or exceed the life-expectancy of the animal. In these cases, pilot experiments are unfeasible and experimental output is generally qualitative, therefore group sizes of 10-20 will be used. This allows for sufficient surviving animals at the end of the experiment to provide adequate tissue for laboratory analysis. Large scale amplification of particular prion strains for biochemical studies involves larger numbers of mice (up to 100) that are required in order to isolate purified prions in the microgram quantities required for structural analyses. In these instances group sizes will be determined by the amount of tissue required rather than by power calculation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will ensure that animals are bred to requirements, and that all animals left on the shelf have a justifiable reason for being kept. Whenever possible we will use the Scrapie Cell Assay developed in the Unit as a method of choice so that our use of mice in bioassays will be reduced significantly.
Furthermore, we have made significant advances in developing new cell lines that can propagate human prions for the first time, albeit to a limited extent at present. We anticipate that when these new cell lines come on stream, even fewer human PrP transgenic mice will be required for routine bioassays.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 stages of the transgenic production process will be constantly monitored and any potential inefficiency likely to lead to excessive use of animals will be dealt with without delay. If any deleterious phenotypes emerge from homozygous lines, such lines will be maintained as heterozygous lines.

We will use improved gonadotrophins to maximise egg yields from superovulated female donors, and females of a minimum age (4 weeks) and/or weight (15 g) will be used to reduce the risk of injury from larger stud males. Analgesics e.g. Carprofen will be administered to recipient and vasectomised mice before, during and post-surgery.

Protocols 8, 10-13 (which would ordinarily be mild) are assigned moderate severity limits because some of the mice in these other protocols will be infected with prions. It is anticipated that the severe threshold may be reached in up to 2% of the animals used, but animals will not be deliberately allowed to progress to this stage. By using anaesthesia and pain killers as necessary, and through careful monitoring, animal suffering will be

reduced to an absolute minimum. We will minimise harm by implementing high standards of care for each animal, by ensuring that the maximum severity for all protocols is either mild or moderate and by using well-defined humane end-points.

We are in the process of acquiring compact behavioural equipment called IntelliCages that are designed to allow mice to be studied in a home cage for the duration of the observation period for any intelligence and/or memory deficits, without human intervention. The resulting reduction in the release of stress hormones ensures that reliable data can be collected automatically from a smaller number of mice, and also ensure the repeatability of the data.

Why can't you use animals that are less sentient?

Whenever possible we do use cell culture assays for our studies, but there are still some aspects of human prion disease research that are only possible in animal models. For instance, fly models of prion disease are available, but this model cannot faithfully recapitulate the neuropathological hallmark of vCJD, that of abundant florid plaques seen in the brain of humans. This distinguishing disease phenotype has to-date only been demonstrated in transgenic mice that are specifically homozygous for PRNP (human prion protein gene) polymorphic residue 129.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

By using anaesthesia and pain killers as necessary, and through careful monitoring, animal suffering will be reduced to an absolute minimum. We will also minimise harm by implementing high standards of care for each animal, by ensuring that the maximum severity for all protocols is either mild or moderate and by using well-defined humane end-points. The acquisition of a new behavioural equipment that allows the behaviour of mice to be studied in their home cage and in social groupings for the duration of the experiment represents a significant refinement. The IntelliCage used together with a detachable Social Box, will allow the following behavioural parameters be measured automatically without operator intervention: territoriality, bonding, dominance, social structure, spatial preference/avoidance and novel object recognition. This minimal handling will result in less stress in the animals and is likely to result in more reliable data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to follow the NC3Rs' ARRIVE Guidelines 2.0, and LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.ubs.admin.cam.ac.uk/files/lasa_aseptic_surg.pdf) as best practice, to ensure that our experiments are conducted in the most refined way. In addition, we will adhere to the "Standard Dosing Information" inserted in this PPL.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

In keeping with the Unit's commitment to the 3Rs promotion, a 3Rs Champion has been appointed to lead our efforts to comply with all aspects of the 3Rs. The 3Rs are a regular feature of the meetings of the AWERB and local Animal Research Scientific Committee (ARSC) which both monitor and ensure that animals are bred to requirements, and that all

animals left on the shelf have a justifiable reason for being kept. The ARSC meets once a month and the 3Rs Champion, who monitors information coming out of the NC3Rs and other relevant sources, has the opportunity to report on 3Rs advances that can be implemented in our practices during the duration of the project.

134. Endothelial-stromal interactions in the control of adipocyte metabolism and browning

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

Metabolism, Adipocyte, Endothelial cell

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?

Having identified certain proteins that are key in controlling whether cells develop into white fat cells (fat storing) or brown fat cells (fat burning), the aim of this project is to establish whether these proteins can be blocked using drugs, in order to promote adipocyte differentiation into brown fat cells, to treat metabolic 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?

Metabolic disease encompasses the following main components: Abdominal obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance and glucose intolerance. Presence of these features put patients in a proinflammatory and prothrombotic state, leading to specific pathological conditions such as diabetes and heart disease, which have a huge health burden throughout the world. In the USA alone, the

prevalence of metabolic syndrome is ~20 % in adults aged 20-39, rising to ~50 % in adults aged over 60. According to the British Heart Foundation, in the UK there is 1 stroke every 5 minutes and 450 deaths every day from heart or circulatory disease, with more than 100,000 hospital admissions each year due to heart attacks. Additional preventative treatments are required to reduce the health burden as well as the morbidity and mortality caused by metabolic disease.

What outputs do you think you will see at the end of this project?

The primary goal of this project is to test whether identified proteins, produced by blood vessel cells and fat cells, affect energy consumption and metabolic health in mice. We will publish the results of these findings in open access journals where they can be viewed by the scientific community and the public. We will also report these findings at scientific conferences. In addition, proteins that we identify as having potential as therapeutic drug targets will be considered for patents to allow discussions with pharmaceutical companies about the possibility of translating these findings into therapies for metabolic disorders.

Who or what will benefit from these outputs, and how?

Short term (1-3 years): The research conducted in this project will be published, to progress scientific knowledge about maintaining a healthy metabolism, increasing the knowledge-base and contributing to our understanding of this important area of biology. We are extremely confident in achieving this outcome.

Short to medium term (3-7 years): The data collected during this project may reveal potential drug targets. If so, these will be patented and discussions will begin to progress the research towards the clinic.

Longer term (> 7 years): Any identified drug targets may, if successfully validated, result in treatments for metabolic disease.

How will you look to maximise the outputs of this work?

We will publish only in Open Access journals (no pay wall) to ensure that the knowledge gained from these studies is available to all.

We will present the work at specialist scientific conferences to ensure that the advancements are disseminated to the field.

We will strive to disseminate both successful (positive) and unsuccessful (negative) results from this work, to ensure that the work is not replicated unnecessarily by other research groups.

If promising drug targets are identified, we will work to progress the research in order to increase the likelihood of therapeutics being developed off the back of our findings.

Species and numbers of animals expected to be used

- Mice: 1000 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.

To test the efficacy of our identified target drugs, we must use living mammals that have similar metabolic processes to humans. Metabolic syndrome in humans is characterised by abdominal obesity, high blood sugar and high serum triglycerides. This phenotype occurs due initially to increased absorption and storage of energy from the diet as fat. The complex interplay between the various fat depots in the body (both white and brown fat), the liver, the pancreas, the endocrine systems and general mitochondrial dysfunction, all contribute to the resulting proinflammatory and prothrombotic phenotype that we need to study in order to achieve our stated aims.

In order to model this syndrome, we need to use a mammalian organism. Mice have been widely used in studies similar to this, and are therefore well-characterised, and we can build on existing data to inform our studies. We need to use knockout organisms to explore the role of identified genes in the syndrome, and the relevant genetically modified mice are available to us. In addition, mice are considered to be the least sentient mammalian laboratory animal.

Adult mice are required because this most accurately replicates the life stage of humans affected by the condition and logistically.

Typically, what will be done to an animal used in your project?

Typically, adult mice will be fed either a normal mouse diet or a diet that is higher in fats (a so-called Western diet) for up to 13weeks.

We will assess the metabolic rate of the mice using a specially-designed cage called the PhenoMaster, which measures the amount of carbon dioxide breathed out compared to the amount of oxygen breathed in. This allows us to calculate the metabolic rate of each mouse. To achieve this, mice must be housed on their own, for a period of up to 4 days at a time, before being returned to their cage mates.
This step will be repeated a maximum of twice during the 13 week long experiment. Mice will be acclimatised to the PhenoMaster cages for 48 hours with their cage mates prior to single-housing, in order to reduce the stress of this novel environment.

In addition, some mice may receive injections of drugs or control agent. We do not expect that these drugs will cause any adverse effects to the mouse but there will be transient pain at the injection site. In most cases just one injection will be given to each mouse in its lifetime. If our findings from these experiments suggest that our drug target is likely to be effective, we will progress to a longer-term experiment where two subcutaneous or intraperitoneal injections per week will be given for a duration of up to 4 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice given a Western (high fat) diet will gain weight during the 13 week feeding period.

The high fat diet given causes greasiness of the skin, which can lead to over-grooming in some instances. To reduce this, previous studies have identified that putting food on the

cage floor (rather than in the food hopper) limits the amount of food dust falling on to the fur, significantly reducing the frequency and severity of excessive grooming behaviour.

Mice will experience some stress from single-housing and from the novel environment of the PhenoMaster.

Mice will experience transient (but not lasting) pain at injection sites.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals (~60 %) will experience the stress of the novel environment and single housing in the PhenoMaster, a maximum of twice in their lifetimes, and no additional procedures. This constitutes a mild lifetime severity.

The remaining ~40 % of mice will also experience injections. Where repeated injections are necessary, these mice will experience a moderate lifetime 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 interactions that we are investigating take place between endothelial cells (blood vessel lining cells) and adipocytes (fat cells). Organoid cultures are under development for a number of tissue types, including fat pads, however these tissues do not exist or function in isolation. The interplay between the endothelial cells and adipocytes are crucial but must be seen in the context of the endocrine, hormonal and physiological interactions that occur between the blood, fat pads, liver, gut and pancreas. We have now completed as much research as is possible in cell culture and using *ex vivo* samples and we now need to establish whether or not the proteins/drugs that we have identified will be effective at improving metabolism in a whole mammal.

Which non-animal alternatives did you consider for use in this project?

We have already used cell culture and *ex vivo* methods prior to embarking on this project. We are now at the stage where we need to test whether the protein interaction that we have identified has a meaningful effect on a whole organism, in order to assess whether or not it would be possible to target these proteins with drugs to treat metabolic disorders.

Why were they not suitable?

There are no alternative models available that allow measurement of global metabolism in

a living organism. Whilst organoid cultures are improving all the time, we are studying not just the effect on adipocytes, but the effect on metabolic disease, a multi-faceted syndrome that manifests as abdominal obesity, high blood sugar and high serum triglycerides as well as raised inflammatory markers and atherosclerosis. Our primary outcome measure is global metabolic rate, which is a composite on a myriad of factors, which cannot be replicated by *in vitro* studies alone.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 methods required for this study have been used here for many years, which allows us to confidently estimate the variability/reproducibility of the data and to make reasonable estimates as to the effect size expected in treatment groups.

Experience of colony management has informed our decisions about the expected number of colony animals required to produce the required cohorts of experimental animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have performed preliminary experiments and the data generated from these has enabled us to estimate the degree of effect that we expect to see and the biological variability in the system. This has allowed us to perform power calculations, that inform us that 13 mice per group is required to see an effect, ifthere is one to see.

In the case of experiments that assess the effect of genetic deletion on the phenotype seen: To reduce the variability between genetically modified animals, and ensure that any effect seen is due to the gene of interest and not external variables/genetic drift, we will use littermate controls wherever possible.
This allows for complete blinding of the experiment as only the researcher in receipt of the genotyping results will know which animals are genetically altered. Wherever possible, unblinding of the PIL carrying out the study will only occur once all data has been collected.

In the case of experiments where an active substance (antibody or small molecule drug) is being compared to a control substance for efficacy: Mice will be randomised using the randomise tool in Excel (or similar) to either group A or B. Another researcher will aliquot the drug or control substance into bags labelled A or B, but, wherever possible, the PIL carrying out the study will have no knowledge of which is the control or active substance until all the data has been collected.

Given that the primary outcome measures generated from this study (body weight, metabolic rate, serum triglyceride levels) are quantitative, rather than qualitative, the risk of bias is also somewhat reduced.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The PhenoMaster system records multiple measurements from each animal over the course of 4 days. This repeated measurement allows a reduced number of animals to be used compared to single measurements.

Following humane killing, multiple tissues (all fat pads/depots, liver, kidney, muscle, bone) will be taken from the mice and assessed to ensure that the maximum possible information is gained per animal. We anticipate that there will not be many surplus tissues, however any that are available may be shared with other groups as required.

One control group (wildtype animals or control drug) will be used as a control for multiple treatments wherever this is possible, to avoid repetition of experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be fed either standard mouse diet or a well-tolerated diet that is high in fats. There is no evidence that these diets cause suffering or distress. In some cases the coats of mice on a high fat diet become greasy, which can lead mice to over-groom themselves, causing sores. Previous studies have identified that part of the cause of greasy coat is dust from the high fat diet pellets falling onto mouse fur from the food hopper. To prevent this, food will be placed on the cage floor, which has been found to prevent much of the over-grooming seen with this protocol. We will closely monitor mice to ensure that if sores do occur they are identified and treated rapidly. Where treatment does not resolve the problem rapidly, mice will be culled by a schedule 1 method, on the advice of the Vet/NACWO.

Metabolism will be monitored using PhenoMaster cages, which measure the metabolism (amount of carbon dioxide exhaled compared to oxygen inhaled) extremely accurately every few minutes. The large amount of data resulting from these cages means that mice can be housed for a shorter period to give conclusive results. The mice may find the PhenoMaster cages distressing at first because of the unusual environment and noise. To reduce this we always ensure that mice are placed in the cages for at least 48 hours with their cage-mates prior to starting the experiment, to allow them to acclimatise.

Injections may be required for some experiments. We will always use the lowest number of injections possible to achieve the required dose.

Why can’t you use animals that are less sentient?

Mice are considered to be the least sentient mammalian laboratory organism. We need to use a mammal for these experiments as we need to replicate the human situation (warm-

blooded mammal with white and brown fat deposits and a complex interaction between these and the liver, pancreas, gut and blood).

An adult life stage is required because we need to monitor global metabolic rate during both activity (night time for mice) and whilst at rest (day time) on individual mice. To achieve this the mice are housed for 4 days and 4 nights (96 hours) to obtain enough data points to account for the variability seen. Clearly this would not be possible with pups as they cannot be separated from their mother for any length of time. In addition, the ratio of brown fat to white fat in infants is very different to that seen in adults, the life stage where metabolic disease affects humans.

Other lower experimental organisms, such as drosophila or zebrafish are cold-blooded and therefore do not have heat-generating brown fat deposits. Given that this project is aimed at increasing the browning of white adipose tissue in order to increase metabolic health, it is crucial that the model organism used has all of the required characteristics if the experiments are to generate meaningful outputs.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Although single housing of mice in an unfamiliar environment (PhenoMaster) is unavoidable scientifically, we have refined the protocol as much as possible by ensuring that cage-mate groups are acclimatised to the environment together, prior to single housing. During the 96 hours of the experiment, mice are carefully monitored, and any that fail to feed adequately (consumption of < 0.5 g food/night or < 0.5 ml water/night) will be removed from the experiment and rehoused in their original cages.

We will continue to monitor the literature for novel refinements and we communicate routinely with the NACWO and NVS to ensure that our procedures are regularly reviewed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We ensure that all our work is published in adherence to the ARRIVE reporting guidelines for animal research and keep a close eye on the literature to ensure that our methods are state-of-the-art. All work will be in carried out with reference to and in accordance with the LASA and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My group and I have attended, and will continue to attend, 3Rs regional conferences and local events and are signed up to receive the NC3Rs newsletter.

Home Office

135. Glioblastoma, Initiation and Recurrence

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain Cancer, Glioblastoma, Brain cells, Invasion, Resistance, Therapies

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?

To understand the biology of the most common, and most lethal, form of brain cancer, glioblastoma. Specifically, to understand how tumours originate from normal brain cells and what mechanisms lead to the devastating recurrence of the disease following therapeutic interventions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

More than 11,000 people are diagnosed with brain cancer each year in the UK (NHS 2019) of which glioblastoma is both the most common and most lethal. Brain cancer carries significant socio-economic burden, which has been estimated at £578 million per year among working age people, the third highest of all cancer types. Tragically, there are currently no effective therapeutic options for glioblastoma, resulting in invariable recurrence of the disease following treatment and a median survival of just 15-17 months. One of the major obstacles to a cure is our poor understanding of the biology of the disease, particularly the mechanisms that underlie tumour initiation and therapy resistance. By tackling these fundamental questions, this work has the potential to identify new treatment strategies for the prevention and more effective treatment of glioblastoma.

What outputs do you think you will see at the end of this project?

Publication of new scientific knowledge furthering our understanding of the biology of glioblastoma in high impact peer-reviewed journals.

New knowledge about how glioblastomas form and what makes them recur. New targets and treatment strategies for glioblastoma prevention and treatment.
Data sets providing detailed descriptions of the molecular makeup of glioblastoma matched precisely to visual images of the tumours will be generated and made available to the scientific community.

Tumour models will be made freely available for distribution to the wider scientific community. Tumour cell lines from human patients.

Who or what will benefit from these outputs, and how?

This work has important and wide-ranging implications for brain cancer, in particular glioblastoma, and its treatment. It will provide new understanding of how glioblastoma develops and recurs.

Our findings will be presented at national and international conferences and published in academic journals. The work will be of interest to a broad audience of pre-clinical and clinical scientists.

Refinements and best practice in surgical techniques will be published. All mouse models and materials will be made available to the scientific community following publication.

Longer-term this work has the potential to identify novel therapeutic targets and/or strategies for the prevention and treatment of glioblastoma. The proposed work is of significant clinical relevance and broad potential social, health and economic impact.

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. The tumour models used will be made available to the brain tumour community.

Many of the grants that are funding this work are collaborative ventures within the university and external collaborations at a national and international level.

Species and numbers of animals expected to be used

- Mice: 17,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are used in this project as they represent the most suitable species for these studies, which cannot be undertaken in humans.

We will produce brain tumour models using mice pups in which cancer-causing mutations will be introduced. We will also induce tumours in adult mice either by introducing mutations in mouse brain cells or by injecting tumour cells into the brain.

Mice are the ideal model animal for these studies as the mouse brain provides a similar level of complexity to the human brain. Many of the specific mouse strains that we will use have already been developed and characterised.

Many of our studies are carried out on brain cells isolated from mice. For these studies we use brains of mice collected just after birth (from day 1 – day 14). At this stage the brain is still relatively immature and can be grown in the laboratory more easily than cells from older brains.

Typically, what will be done to an animal used in your project?

Mice will undergo two main surgical procedures: 1) they will be given brain tumours; 2) they will be subjected to brain injury. This will allow us to study how tumours form and recur after treatment and to identify new treatments for glioblastoma.

To produce tumours, mouse pup brains will be injected with chemicals that cause brain cancer. Alternatively, tumour cells from patients or mice will be injected into the brain of adult mice. In both approaches, injections will be performed under general anaesthesia. For brain injury experiments a thin needle will be inserted directly into the brain tissue through a small hole drilled in the skull under general anaesthesia.

Tumour development will be followed by imaging methods like MRI scanning under general anaesthesia. In some mice, an imaging window will be inserted over the tumour to allow visualisation of tumour development and recurrence under a microscope. To model tumour regrowth post-treatment, mice will undergo treatment regimens that mimic the treatments administered to patients. These include surgical tumour removal (resection), chemotherapy, immunotherapy and radiotherapy. In some cases, experiments will require administration of chemicals before, during and/or after the surgical procedures. These chemicals will be delivered by either injection into the body cavity or orally.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals which receive an injection may experience some discomfort, but this is unlikely to last.

Animals which undergo surgical procedures may experience post-operative complications. These will be monitored, and pre- and post-surgical pain killers will be given.

Mice with brain tumours may begin to lose weight. Some mice become aggressive or may show unusual behaviours as the tumours develop and in some cases mice can have seizures. Strict humane endpoints are in place based on weight loss and change of behaviour and if any of these end points is met, mice will be humanely killed.

Any animal displaying signs of pain indicated by hunched posture, immobility, or hair standing on end, 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)?

Around 30% of the animals in this project will be genetically altered breeding mice which will provide pups for the preparation of cells and brain slices. These are mild severity.

The remaining 70% will be classified as moderate. These are mice which will undergo some form of surgery under general anaesthesia or are mice which have been injected with substances.
Development of brain tumours is classed as moderate as it can be associated with weight loss, loss of body condition, changes in behaviour and in some cases mild seizures. Any sign of these occurring and animals will be monitored daily and humanely killed prior to, or once, humane endpoints are reached.

What will happen to animals at the end of this project?

- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Tumour development is a complex process that relies on the interactions of many types of cells. This cannot be satisfactorily modelled in cells in the laboratory. Therefore, it is necessary to use animal models. It is possible to isolate some of the individual brain cells and grow them in the laboratory to study their interactions. It is also possible to use brain slices to understand how tumour cells invade into the brain. We have used both approaches successfully in the past and will continue to do so. However, as successful as these models are, they cannot fully reproduce the biology of the tumour and the surrounding brain tissue as well as its interactions with the immune system, which play a crucial role in the disease.

Which non-animal alternatives did you consider for use in this project?

The use of mini organs grown in a dish (organoid cultures), is a growing field and is being employed as a suitable replacement for animal studies. It has been used successfully to model many cancers including colorectal, oesophageal, liver and lung cancers. Brain organoids, known as “minibrains” can also be generated and have been used successfully to model some brain diseases (e.g, schizophrenia). These could offer an alternative method for investigating brain cancers.

Why were they not suitable?

Despite continued improvements in the technologies for culturing brain organoids from human stem cells or human brain tumour cells they are unsuitable for this project. This is because they lack many of the cell types that are important for tumour development and regrowth, such as blood vessels, immune cells and white matter. In the absence of these structures, the key processes that underlie brain tumour progression and regrowth following treatment cannot be studied.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates of the numbers of animals used are based on previous experience and statistical methods. For tumour studies this corresponds to 8-10 animals per treatment group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our extensive use of cells and brain slices limits the number of animals required for this project. In addition, for each experiment consideration is given to ways of reducing the number of animals needed. Our protocols are designed to obtain the maximum possible data from each animal (i.e. combining imaging of the live mouse brain over time with analysis of the tissue post-mortem).

Mouse lines that are not required for extended periods will be frozen and stored rather than maintaining live animals unnecessarily. Prior to generating new genetically altered mice we will ensure that these lines do not already exist by searching databases and publications.

To reduce animal numbers used, we will consult with statistical experts within the institute. In addition, we will use the NC3Rs ARRIVE guidelines in reporting our results, and the NC3Rs Experimental Design Assistant in considering the design of our experiments (e.g., allocation of animals to groups, blinding).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To optimise the number of animals used in this project we will follow strict breeding programmes to produce only those animals that are required. As we use animals of both sexes wastage from litters will be minimised. Similar genetically modified mice are used across many different projects, maximising the usage of these lines.

To minimise the numbers of mice used for tumour studies, efficacy of treatments will be first tested on cells in the laboratory prior to testing them in the mice. The use of many different tumour imaging methods will also maximises the amount of data collected from each mouse.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

These studies involve mice, which are the simplest mammal with the necessary brain complexity to be able to accurately model these cancers. Altering genes in mice is relatively simple and most of the lines that we will use in this project are already available.

We intend to perform the following procedures of moderate severity: brain injury, brain injections, brain tumour treatment (including tumour removal (resection), chemo-, radio- and immuno-therapy) and imaging studies. These are all well-established methods, which in our experience produce minimal discomfort and have negligible infection rates. All the necessary surgical techniques are already in place in the research group and are continually being refined with the NACWOs and NVSs. Humane endpoints are strictly adhered to and designed to minimise suffering and lasting harm.

All animals undergoing general anaesthesia are provided with the appropriate pain relief during and after procedures. They are also provided with easy access to recovery fluids and easily eaten food (wet mash and pain relief jellies).

Why can’t you use animals that are less sentient?
We use pups to prepare cells and brain slices. At these stages the brain is not fully developed making the animals less sentient. This minimises the use of adult mice for our studies.

For tumour studies, the use of immature stages or less sentient animals would yield little information, as glioblastoma is an adult tumour in humans. In our models brain tumours develop over months and become apparent at adult stages, making it the most realistic model of human brain cancer development.

For a proportion of mice brains will be collected for post-mortem analysis of brain tumours under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will consult with NVSs, NACWOs and the staff for any local refinements in surgical anaesthesia and analgesia as advances are introduced. Training of staff is paramount, and we have many members of the team who have extensive experience in the surgical techniques that we use. For novel techniques (such as X-ray irradiation or MRI) we are fortunate to be collaborating with experts and innovators in those fields and we will draw on their expertise.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs guidelines and ARRIVE 2019 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive updates from the NC3Rs website (nc3rs.org.uk) which publish a monthly newsletter with information on regulatory changes, policy and new technologies and approaches for managing the 3Rs in animal research. We regularly consult the ARRIVE 2019 guidelines and use the online facilities available from Charles River to continually monitor and refine our protocols to satisfy the 3Rs.

136. Evaluation of 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

Key words

cancer, therapy, ionising radiation, normal tissue

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to identify novel therapeutics to improve the clinical management of 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?

Despite advances in cancer therapy, clinical benefit is limited by lack of response, development of resistance, and normal tissue toxicity. This work will enable us:

- To identify novel drugs that can significantly improve the efficacy of current therapies, such as radiotherapy and chemotherapy.
- To understand the biological impact of novel therapies on both tumour and normal tissues. To identify which patients would benefit most from novel anti-cancer treatments.

What outputs do you think you will see at the end of this project?

- Identify new agents that can improve the efficacy of current anti-cancer therapies, such as radiotherapy and chemotherapy.
- Identify new targets for anti-cancer therapies.
- Generate knowledge on how new agents or new targets selectively enhance the killing of tumour cells.
- Publish data in peer-reviewed journals and present data at conferences. Discuss with clinicians to initiate clinical trials.

Who or what will benefit from these outputs, and how?

Short term:

- Basic Oncology scientists and clinical oncologists will benefit from advancement of basic oncology knowledge and improvement in current cancer treatment through publications and conference presentations.
- Drug development scientists will benefit from identification of novel drug targets for potential cancer treatment through publications and conference presentations.

Long term:

- Cancer patients will benefit from (i) personalised anti-cancer therapies, (ii) the guidance on combining the new agents with current treatments in the clinic, and (iii) the scientific rationale for clinical trials.

How will you look to maximise the outputs of this work?

- We have established wide collaborations with academia and industries, which will accelerate the project development and strengthen the analysis of findings from this project.
- We will share technique refinements via 3Rs subcommittee. We have set up an ex vivo technique, and will apply for NC3Rs funding to validate this approach in comparison to in vivo models.
- We will publish data in the most widely read scientific journals.
- We will also include any negative data in publications to avoid unnecessary duplication.

Species and numbers of animals expected to be used

- Mice: 3900 mice over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice will be used, because

- Our interest is adult human tumours. Our tumour models generated in adult mice closely resemble the human cancer.
- The mouse represents the lowest vertebrate that most accurately reflects the human physiology and the immune system for cancer research.
- Adult mice are more able to tolerate the side effects of treatments compared to younger mice.

Typically, what will be done to an animal used in your project?

Typically, tumour cells will be injected under the mouse skin to grow subcutaneous tumours or into mammary fat pad to grow orthotopic breast tumours or into the lung or the tail vein to grow orthotopic lung tumours. In some cases, the carcinogen urethane will be injected into the abdomen to induce lung tumour development. When tumours are formed, mice will be treated with test agents (usually by mouth or injection) and/ or viruses (typically, recombinant adeno-associated viruses by injection) and/ or local tumour radiation to study the effects on tumour growth and the response of tumour and non-tumour cells. Mice may have blood samples taken either from the tail vein or from the heart when animals are killed humanely.

The superficial tumour model will be studied for up to 10 weeks after tumour cell implantation. The lung tumour model generated by injection of tumour cells into the lung or the tail will be studied for up to 4 weeks.The urethane-induced tumour model will be studied for up to 34 weeks after urethane injection.

What are the expected impacts and/or adverse effects for the animals during your project?

- Some animals in this project may experience moderate adverse events as follows: Superficial tumours may cause ulceration, intramuscular growth, and weight loss. Urethane-induced lung tumour growth may cause weight loss and breathing difficulty.
- The growth of orthotopic lung tumour generated by intrathoracic or intravenous injection of tumour cells.
- Administration of substances may cause body weight loss, diarrhoea, and changes in behaviour and appearance.
- Local radiation may cause skin irritation, ulceration, and weight loss. Non-invasive imaging may cause failure to recover from anaesthesia.
- Typically, animals will be killed if adverse effects last up to 48h, depending on the 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)?

Overall in this licence, mild severity: 52%, moderate severity: 48%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 is to identify novel therapeutics to improve the efficacy of current cancer treatment. Tumour cells and different types of non-tumour cells constitute the "tumour microenvironment", which is crucial to tumour growth and response to therapies. However, cultured cells do not adequately recapitulate the cellular context and interactions in which cancers evolve. In addition, the body alters the chemical nature of drugs (metabolism) in a way which is difficult to predict from cell culture studies, but which is essential to know if we are considering taking these drugs into patients. Therefore, it is necessary to perform these studies in animal models.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives (cell/tissue/organoid cultures, and human tissue analysis) were considered first to identify the most suitable candidate compounds and combination treatments for further evaluation in animal models.

Why were they not suitable?

Non-animal alternatives cannot gain information on the activity, distribution, and metabolism of drugs inside a living organism, and they cannot mimic the complex tumour environment, and the interaction between tumour and non-tumour cells in the body. Therefore, they cannot substitute for 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?

Data from a pilot study will be used to determine the sample size for a definitive experiment. Typically, 5-6 animals/ group will be used to obtain an estimate of the variability of the efficacy measurement (e.g. tumour volume or tumour incidence). Then power calculations will be used to estimate the number of subjects per group required to demonstrate a significant treatment effect.

For the majority of tumour models, group sizes are anticipated to be in the range of 6-10 animals. Based on our past experience, group sizes of 6-7 animals are usually sufficient to identify the most active therapies.

What steps did you take during the experimental design phase to reduce the

number of animals being used in this project?

- We will design our experiments following the NC3R's Experimental Design guidelines, including determining sample size by power analysis, and ensure randomisation and blinding in experiments. Furthermore, additional resources may be used to aid experimental design such as the NC3Rs experimental design assistant tool.
- Wherever possible, single sex (female) and inbred strains of mice will be used to minimise inter- animal variability.
- Wherever possible, the design of experiments will involve factorial design (e.g. treatment, dose size, dose duration, and tumour size) to maximise information gained from a minimal 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?

- Pilot studies will be performed to gain information on tumour response to therapies and statistical variability. They will then be combined with G-power analysis to determine the minimum animal number required for achieving a statistically significant result.
- For each experimental animal, multiple parameters will be evaluated to obtain as much information as possible.
- Live imaging of the same animal at multiple time points will reduce the numbers required.
- Where possible, mouse tissues will be shared among the research group. For example, some tissues can be used for other 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.

- Subcutaneous tumour models are generated by injection of tumour cells under mouse skin under brief general anaesthesia. This is a refined method for tumour induction, as subcutaneous tumours usually do not invade normal organs. Tumour growth can be measured by callipers that do not affect the welfare of animals.
- The urethane-induced tumour model is established in urethane susceptible mice by injection of urethane into mouse abdomen. The development of tumours generally follows a predictable course and animals will be killed before experiencing any significant clinical signs.
- The orthotopic breast tumour model is generated by injection of tumour cells into the mammary fat pad under brief general anaesthesia, and breast tumour growth can be measured by callipers that do not affect the welfare of animals. The orthotopic lung tumour model is generated by injection of tumour cells into the lung (under brief general anaesthesia) or into the tail vein. The development of orthotopic lung tumours generally follows a predictable course and animals will be killed before experiencing any significant clinical signs.Tumour models will be administered with test agents alone

or in combination with radiation under general anaesthesia. In some cases, animals will be injected with viruses (typically, recombinant adeno-associated viruses). The safety of administered agents, or viruses, or combination treatments should be tested prior to efficacy studies. Therefore, the adverse effects of the treatments in efficacy studies are expected to be low.

Why can't you use animals that are less sentient?

- The mouse is the lowest order species that can be used to study tumour growth in a way relevant to humans.
- Tumours develop over weeks to months, so use of immature or terminally anaesthetised animals is not practical.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- We will perform pilot experiments on all unfamiliar tumour models and procedures in order to establish appropriate experimental and humane endpoints prior to undertaking larger-scale experiments.
- Tumours will be grown for the minimum time and volume that are needed for achieving scientific outcomes.
- Following treatment, mice will be kept warm and monitored continuously until recovery. The anaesthesia will preferably entail the use of inhalation agents whenever possible.
- If body weight falls beyond 10%, moist palatable food will be provided, and animals will be checked daily.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In order to conduct the experiments in the most refined way, we will follow the published best practice guidance below:

- **Guidance for the welfare and use of animals in cancer research** (*Br. J. Cancer*, 2010, 102: 1555-1557)
- **Preclinical formulations for discovery and toxicology: physicochemical challenges** (*Expert Opin Drug Metab Toxicol*, 2006, 2: 715-731)
- The design of Animal Experiments: Reducing the use of animals in research through better experimental design (By Michael Festing *et al*)
- **Handbook of Laboratory Animal Management and Welfare** (By Sarah Wolfensohn and Maggie Lloyd)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

- We will attend NC3R seminars and University PIL holder meetings.
- We will sign up university's 3Rs newsletter, and use 3R online resources.
- We will update our methodology according to new policies and technologies to minimise welfare cost.

Home Office

137. Translational Tumour Biology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - voidance, 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, Translation, Therapy, Fibrosis

Animal types	Life stages
Mice	neonate, juvenile, adult, 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?

To better understand the molecular and cellular processes that are required for cancers to grow and spread in order that we can generate new therapies that we can take to (ie translate to) 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?

While we have made significant progress in treating some types of cancer successfully (eg certain childhood leukeamias) 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. Added to this we have also established that even within a single tumour in a single person, there are multiple different genetic changes that have occurred while that tumour developed and we are currently unable to say whether a particular therapy will be equally effective for each of these genetic variants. Thus cancer remains an incredibly complex disease that requires detailed scientific research to identify new 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. We are currently testing new drug compounds against novel therapeutic targets we have identified in the lab

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.

We are strong advocates of minimally-invasive imaging (MRI, PET,SPECT). Current studies where such imaging modalities are used for deep-tissue tumour studies (eg pancreatic, lung) suggest that we are improving the scientific analyses of such studies and we hope this will be adopted by other institutions.

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 and imaging tracers. 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 anmal experiments that would otherwise have been conducted will no longer be necessary reducing the numbers of animals used.

We will share directly how non-invasive imaging can improve animal experiments by

improving the scientific interpretation of deep-tissue tumour studies.

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: 27000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are interested in understanding better the processes that lead to human cancers initiating, developing and spreading, in order that we can develop therapeutic strategies that we can translate to the clinic. The mouse has many virtues that mean it is an ideal model for our studies. Thus mice have:

-been used successfully for decades by scientists to develop effective drugs in the clinic.

-a breeding-cycle that is relatively quick

-many genetically altered (GA) versions available to make scientific research efficient, relatively quick, and require fewer animals. The technologies to generate further GA mice that possess more relevant gene modifications is now routine and provided through commercial sources which limits the errors by less experienced staff in making these animals.
With the exception of genotyping, which needs to be done early (usually on neonates, juveniles if repeats required) we are interested in processes that occur mostly in adult humans. Thus we use adult mice for our studies.

Typically, what will be done to an animal used in your project?

All experiments require a pre-planned series of procedures that are designed to maximise the data from the use of the mice, while using the minimum number of mice and imposing the minimum amount of discomfort and harm.

Many of our experiments require mice to undergo repeated anaesthesia, often firstly for a minor surgery to allow implantation of cells. Most therapies that require repeated

administrations are given ip as this seems to be give minimal discomfort and results in rapid accumulation of the compound in the circulation. For most ip injections mice are not anaesthetised, as experienced staff do each injection in a few seconds removing the harm of additional anaesthesia. Repeated tail vein injections are avoided as much as possible as tails can be become inflamed and scabbed with the repeated wounding with a needle.

We use many types of imaging to ensure we monitor the progress and response of tumours. Again these require mice are anaesthetised and injected with either a substrate (luciferin) or a radiotracer (for SPECT or PET studies. MRI does not require an additional injection but does require more skill in interpreting the resulting images.

A common experiment design might be: tumour cells are implanted in the pancreas (week 1), BLI/MRI imaging confirms tumour presence and therapy commences (week 2), therapy is repeated 2-3 times (if ip) per week or once daily for a maximum of 7d if by oral gavage (weeks 3-5). Tumour growth is monitored by imaging (eg BLI) once weekly (weeks 3-8). Mice are terminated as tumour volume (established using either mm^3 determined MRI volumes or calibrated BLI signals from the same MRI analysed mice) approaches/reaches home $1.44mm^3$ total or mouse welfare suggest earlier termination.
Thus many experiments, cohorts of 10 mice (sufficient to detect a 25% change, with statistical significance of 0.05) will last 8-12 weeks at most and mice will receive therapy 3-7d per week for 3-4 weeks, anaesthesia and imaging once per week for the period of the study, commencing one week after tumour implantation. For a simple experiment (drug versus no drug) this requires 20 mice. These data allow temporal changes in tumour growth and spread to be monitored in individual mice over the study period. To achieve the same data quality and quantity without repeated weekly imaging, cohorts of 20 mice would have to be culled at weekly time-points throughout the study.

What are the expected impacts and/or adverse effects for the animals during your project?

Injections give transient pain as they do in humans and mice recover rapidly.

Mice recovering from abdominal surgery will receive analgesia and usually show little change in their general behaviour, mobility feeding or drinking suggesting the discomfort is manageable.

Early tumour growth does not usually cause pain or discomfort. As tumour grows its possible the location causes physical problems (in eating, mobility, blockade of an essential vessel) or it may become ulcerated. In most cases these mice will be immediately killed by schedule 1 unless vet advice suggests the discomfort can be managed with appropriate drugs.

Implanted tumours tend to develop and grow at a more predictable time frame that GA mouse tumours. Thus GA mice experiments often require several months before tumours arise and experiments can be conducted whereas implanted tumours are often completed in 3 months.

Some treatments cause significant weight loss. If the weight loss is progressive and reaches 20% the mice will be killed. However, it may be the weight loss is transient. Thus the bleomycin used to induce lung fibrosis can induce up to 25% weight loss but experience shows that the weight loss is transient and mice recover their weights rapidly

after an initial inflammatory period. These mice provide very valuable data on fibrosis in humans, a fatal disease in 7-8000 people annually in the UK.

Any procedures applied to mice that result in abnormal behaviours that equate to significant harm will result in those mice being 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)?

Moderate severity. Depending on the protocol 10-100% of mice will experience moderate severity.

Specifically, of the maximum 27000 mice that may be used during the life-time of this license, we estimate a maximum of 9350 may 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?

Our goals are 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 as closely as possible match the complexity of human cancer including have 1) typical support cells such as fibroblasts, macrophages, neutrophils 2) typical proteins in the immediate environemnt that are specific for a particular organ 3) a blood supply to enable the cancer cells to migrate to distant sites and metastasise but also so that we can deliver experimental compounds to the tumour.

There are no systems in the laboratory that can achieve 1-3 except a live animal. Thus animals are essential to our programme.

Which non-animal alternatives did you consider for use in this project?

Our laboratory have been developing 3D organotypic tumour mimic models in the lab for over 10 years that allow us to address discrete questions so that we dont always need to use animals. Essentailly we combine commercially purchased proteins typical of most organs eg collagen, and prepare collagen gels into and onto which we place cancer cells and other tumour assoc iated cells. We have used this laboratory 3D models extensively to analyse how fibroblasts affect the growth and invasive behaviour of cancer cells. We then use our observations to develop more refined animal experiments. Our methods have been shared across the world and our now a standard for most laboratories studying tumour biology.

Why were they not suitable?

The laboratory systems we have designed are extremely valuable and can address discrete defined questions and generate valuable knowledge. However the will never fully recapitualte the complexity of a live tisuue 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.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Yes. These numbers represent estimated maximum mouse usage based on our current usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

25 years of experience in designing, running and publishing in vivo studies means I am well prepared for training and supervising my staff in effective means of designing and executing in vivo studies.

First we start with a clear hypothesis. This is essential so that we can design an experiment with defined goals, sufficient numbers of mice, a clear monitoring strategy, a pre-planned means of analysis, a randomisation plan and a data blinding plan. All of this should be defined and agreed BEFORE the experiment commences.

For those experiments where a therapeutic change is desired, we would first conduct multiple in vitro studies including, if possible, 3D multicellular pathology-mimicking models in order to prove that indeed, there is a potentially useful biological effect. Only then would in vivo experiments be considered. Sometimes the justification may come from studies from other laboratories. The level of effect helps to determine the expected % biological change that will inform the power calculations.

For many years we have used successfully the on-line power calculation tool (http://biomath.info/power/). Our resulting experiments have produced data that provide statistical significance and thus more scientific value.

We utilise multiple different imaging tools to monitor tumour progression and response. This allows us to follow temporally the change in tumour activity during an experiment without the need to kill the animal. These tools include bioluminescence, MRI and radio-imaging (SPECT/CT and PET/CT).

In preparation for this license I have become familiar with the excellent NC3Rs EDA tools. I will be introducing these tools to all of my team with the instruction they should become familiar and where valuable, to use them in their experimental designs. The NC3R EDA

tools can improve experimental design and increase my staff's knowledge and understanding of in vivo studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where the scientific question requires modification of gene expression in a target tissue, whenever possible we will always utilise GA mice designed such that there is a minimum number of crosses with other mice to achieve the desired genotype. We have already rejected a GA mouse design that has a desirable genotype but which itself is generated from three different GA mice. Since we wish this final genotype to be expressed on a cancer background where there may already be two GA crosses involved, the wastage in animal life to achieve effective genotypes was considered too high to accept this experimental design.

Pilot studies are essential to minimise the use of mice in our studies as this will allow us to know with a high degree of confidence the correct number cells to form tumours, the time frame for tumours to grow, whether the tumours spread, the correct dose of drug to achieve the maximum biological effect, the correct dosing to achieve optimal pharmacokinetics. When these facts are in place we can accurately design good experiments to address our questions.

We have indicated that we are keen that spare tissues from our studies are made available for others to use as a means of performing either pilot or even experiments, without the need to use additional mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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) 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. In mice that develop chronic inflammation (eg Vav-Cre+ve/itgb8$^{fl/fl}$) mice are used before the disease causes significant discomfort (approximately 12-16 weeks).

Mice bearing transplanted tumour cells/tissues will be used: Cells or tissues derived from mouse or human tumours or circulating tumour cells will be injected or implanted into mice subcutaneously (sc), orthotopically or intravenously (iv) as required by the scientific

question. Experienced handlers will inject the sc and iv cells/tissues minimising pain and discomfort to mice. Mice will require anaesthesia and surgery for orthotopic injections and implantations of organotypic gels 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 minmise pain and harm to animals. In some studies it will be necessary to cause fibrosis in the lungs or liver through introduction of an irritant such as bleomycin, carbon tetrachloride, respectively. These mice will be used before the fibrosis advances to a severity that causes significant discomfort.

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 local trained staff and as required, the vet.

Why can’t you use animals that are less sentient?

Most of our experiments require that inflammation, fibrosis or tumours will develop over a time period. This is because the pathologies are a result of complex interactions between live cells. Thus it is not possible to use animals that are terminally anaesthetised. We also need to use a simple mammal in order to as 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 MRI and other minimally-invasive imaging methods to improve the analysis and monitoring of deep tissue cancers that develop either through orthotopic implant or growth 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 MRI protocol has had the immediate benefit that there is limited chance that an animal develops an internal tumour that exceeds the specified maximum size limits of the license.

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 also use the NC3R EDA tools on line.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute regulalrly hosts updates in experimental animal usage, including

developemnts in 3Rs that I try to attend or send representatives to from my team. I also regulalrly attend (and have spoken at) NC3R events to hear how other researchers conduct their research.

138. Regulation of breathing and oxygen supply by AMPK-dependent signalling pathways: from pulmonary hypertension to sleep apnoea

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

AMP-activated protein kinase, hypoxia, breathing, pulmonary hypertension, sleep apnoea

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

At altitude a fall in available oxygen increases breathing frequency to supply more oxygen to our lungs. Onward oxygen transfer to the bloodstream is aided by a process, hypoxic pulmonary vasoconstriction, that diverts blood flow to oxygen-rich regions of the lungs by closing blood vessels in oxygen-deprived regions. I have demonstrated that an enzyme, AMPK, supports these reflexes. Moreover, my studies suggest that AMPK deficiency triggers conditions symptomatic of altitude sickness, namely sleep disordered breathing and pulmonary hypertension (high blood pressure in the lung). Obesity and type- 2-diabetes lower AMPK levels and are, like altitude sickness, associated with sleep-disordered breathing and pulmonary hypertension. Therefore, I propose that AMPK deficiency causes sleep disordered breathing and pulmonary hypertension. I aim to determine how AMPK regulates ventilation- perfusion matching at the lung, breathing and thus oxygen supply, and the mechanisms by which AMPK deficiency may precipitate pulmonary hypertension, sleep-disordered breathing and sudden infant death syndrome (SIDS). Our data suggest that sleep apnoea and SIDS are associated with the metabolic syndrome and arise at the brainstem. However, we have yet to pin-point where

dysfunction arises within the brainstem and the molecular, cellular and circuit mechanisms involved.

My future aim is to characterize these mechanisms in more detail in order to identify where and how we may target AMPK to provide for new therapeutic interventions for pulmonary hypertension, sleep- disordered breathing and SIDS.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

Our findings challenge current dogma in many respects and, despite significant advances, our understanding of the mechanisms by which AMPK governs cardiorespiratory functions remains rudimentary. Moreover:

Sleep-disordered breathing is associated with all-cause mortality and affects ≈2.5 million males and ≈1 million females (UK Office for National Statistics); U.S.A., ≈15-65 million;

Idiopathic-pulmonary hypertension affects ≈15 per million, life expectancy ≤6 years;

Pulmonary hypertension and sleep-disordered breathing are symptomatic of altitude sickness;

SIDS is the leading cause of death in otherwise healthy infants, and currently accounts for approximately 300 deaths per annum in the UK and 2000 deaths per annum in the USA.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

Wild-type and transgenic mice, approximately 1000 over the course of this 5 year project

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

We will use wild type and transgenic mice to provide in-vitro preparations by schedule 1 that will allow us to study the molecular, cellular and circuit mechanisms that determine the hypoxic ventilatory response, ventilation perfusion-matching in the lung and thus address the causal mechanisms of sleep apnoea and pulmonary hypertension.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

We cannot study the response of complex physiological systems using cells in culture. Therefore, mice are required. Animals used in this project will provide organs, tissues and cells for the development in- vitro models.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Animal use has been assessed by power calculations. Where possible replicates will be taken from tissue samples from single animals in order to reduces usage. Furthermore, we will employ continued use in order to reduce animal numbers where possible

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

Wild-type and transgenic mice will be used to address our research aims. The models to be used are the most refined because they carry cell-specific genetic deficiencies that impact the systems physiology and mirror known pathophysiology (pulmonary hypertension, sleep apnoea). Harm will be minimized by using anaesthesia during and after surgery, terminal anesthesia where possible, and by employing continued use. We will also employ studies using recombinant model systems (cell cultures) to further explore molecular mechanisms where feasible.

Home Office

139. Toxic industrial chemical antidotes

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

cyanide, antidote, pharmacokinetic, therapy, prophylaxis

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 the effectiveness of antidotes against toxic industrial chemicals that cause metabolic poisoning, when the antidotes are administered as prophylactics (before exposure) or as therapeutics (after exposure).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

People may exposed to toxic industrial chemicals (TICs) through accidental exposure, smoke inhalation from a residential fire or deliberate malicious attack. These exposures can be lethal unless treated quickly with an antidote. The TIC that is the main focus for this project is cyanide.

There are antidotes against cyanide that currently have regulatory Marketing Approval.

These have demonstrated effectiveness in animal studies that model cyanide exposure through smoke inhalation, however, a study published in a peer reviewed journal has shown these antidotes are less effective against a larger cyanide exposure malicious attack scenario.

The antidotes that will be researched under this project have demonstrated effectiveness in animal studies that model cyanide exposure through smoke inhalation and may be effective against larger cyanide exposure doses.

What outputs do you think you will see at the end of this project?

New data and information on antidotes against a toxic industrial chemicals will be an output of this project. Other outputs will be the concentration of the antidotes measured in blood samples collected following administration of the antidotes. The effectiveness of antidote doses or the measured antidote concentrations to protect against the lethal effects of toxic industrial chemicals, such as cyanide exposure will also be an output. Additional outputs will be the concentrations of the toxic industrial chemicals measured in blood samples collected following an exposure challenge, which can be used to inform the development of other antidotes or treatments. The data from this project will be published in peer reviewed journals and may be used for the pharmaceutical development of the antidotes towards regulatory authorisation for human use.

Who or what will benefit from these outputs, and how?

The pharmaceutical development of the antidote will have new data from this project, which (if the data show effectiveness) will support the application for authorisation of the antidote as a medicine by a pharmaceutical regulator. In the longer term, the data from this project will provide evidence for the use of the antidote against exposure to toxic industrial chemicals to save lives of individuals who are exposed to these toxic industrial chemicals.

How will you look to maximise the outputs of this work?

Through collaboration and discussion with other researchers working on this or similar antidotes, publication of the model and results in peer reviewed journals.

Species and numbers of animals expected to be used

- Pigs: 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.

It is not ethical to test the effectiveness of antidotes against toxic industrial chemicals such as cyanide in human clinical trials, therefore, the evidence that they are effective must be demonstrated in animal models of exposure to toxic industrial chemicals.

Adult pigs are being used in this project as their similarity in size and physiology to humans makes them the most appropriate. The existing data on the effectiveness of

cyanide antidotes have been obtained in this species, meaning that the results from this project can be considered in relation to that existing data.

Typically, what will be done to an animal used in your project?

Most of the pigs (74% of the total to be used in this licence) will be terminally anaesthetised throughout the experiments (non-recovery severity). Once the pigs are anaesthetised, they will be attached to non- invasive monitoring devices and will be surgically implanted with cannulae in blood vessels. These steps are to enable administration of substances, sampling and monitoring of normal functions, for example heart rate, breathing rate and blood gases. Following a period of baseline physiological data / sample collection, all of these non-recovery pigs will be administered a toxic industrial chemical using the implanted cannulae. Around one quarter of the 74% will be to find out what dose of toxic industrial chemical models human exposure. Around three quarters of the 74% will be to determine the efficacy of antidotes, meaning finding out whether the antidote is likely to save the life of a person exposed to the chemical. For antidote efficacy experiments, pigs will receive either an antidote or a control compound (known to have no efficacy) so that the effect of the antidote can be compared to the control. All pigs will be monitored throughout the experiment, then, at the end of a set experimental period, the pigs will be killed by a Schedule 1 method (overdose of anaesthetic), whilst remaining anaesthetised.

The remaining pigs (26% of the total to be used in this licence) will be surgically prepared with cannulae under recovery anaesthesia as a moderate severity procedure. These pigs will emerge from the anaesthesia, regaining consciousness, and will have a minimum of one week post-operative recovery from the surgery. The pigs will wear jackets and will also be single housed, both the jackets and the single housing aim to protect the cannulae. When single housed the pigs will be able to see, hear and smell other pigs, they will also have environmental enrichment. Following this post-operative recovery period, the pigs will be administered antidotes by either the intravenous route (via the cannulae) or the oral route and blood samples collected (via the cannulae) for measurement of the concentration of that antidote in the blood. Pigs may be tethered to help with the administration of antidote and collection of the blood samples using the cannulae by reducing the need to closely handle and restrain the pigs. At the end of the sampling period the pigs with be killed by a Schedule 1 method (overdose of anaesthesia).

What are the expected impacts and/or adverse effects for the animals during your project?

The pigs that are challenged with a toxic industrial chemical are expected to exhibit the adverse effects associated with that chemical. For example, pigs exposed to cyanide may develop metabolic acidosis as the cyanide stops their cells from using oxygen, they may stop breathing which can cause cardiac disturbances, seizures and can ultimately be lethal. The pigs will not be aware of these adverse effects as they will be anaesthetised and therefore unconscious during the experiment.

Pigs that receive an antidote are not expected to have any adverse effects due to the antidote. The antidotes have previously been used in pigs and the maximum feasible/tolerated doses are known. Adverse effects are not expected from oral administration of antidote. The substance will be mixed with a palatable food or liquid with a flavour that the pigs are known to like and the pig is expected to take the substance voluntarily e.g. by eating it out of a bowl or drinking it from an offered syringe.

Induction of anaesthesia includes some transient distress. This will be minimised through sedation of the pigs before induction of anaesthesia.

The pigs that have surgery followed by post-operative recovery (26% of the total to be used on this licence, as described above) are likely to experience pain following surgical procedures to implant cannulae in blood vessels for several days after surgery. This will be minimised through the administration of pain relief and anti-inflammatory drugs. The risk of infection will be minimised through aseptic technique. Antibiotics will be administered as required. It is likely that some pigs may experience distress from the jackets used to protect implanted cannulae, these will be put on the animals whilst they are anaesthetised to minimise the distress. The jackets have been specially made for our Establishment, they have soft cuffs to reduce rubbing and our experience with these jackets is that they are well tolerated by the pigs. Pigs that have the implanted cannulae will be single housed, the distress and harm from this will be minimised by housing pigs in pens that are near to other pigs, enabling them to see, hear and smell other pigs. They will also have environmental enrichment. The pigs may be attached to a tether for the administration of antidote and collection of blood samples for the measurement of antidote concentrations. This is to minimise the need for close handling and restraint of pigs and the distress associated with that close handling and restraint. Pigs that are tethered, will be tethered for a maximum of six hours in total during the 24 hours study. Tethered animals can move around their pens at will, stand up and lie down as they prefer, the tether has a weighted swivel mechanism that is designed to minimise the impact of tethering on normal locomotion and behaviours.

Expected severity categories and the 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 (74%) of the adult pigs will be of non-recovery severity, these are the animals that will be terminally anaesthetised throughout the protocol. These animals will be challenged with a toxic industrial chemical at a dose that may be lethal. Around one quarter of the 74% will be to find out what dose of toxic industrial chemical models human exposure. Around three quarters of the 74% will be to determine the efficacy of antidotes.

The remaining (26%) adult pigs will be moderate severity, due to the experience of anaesthesia, surgery and post-operative recovery. The pigs will be surgically prepared under anaesthesia, have a post-operative recovery period and then will be administered antidote. This is to investigate the antidote and how it behaves in the body following administration. The information gained about the antidote will be used to select the dose of antidote used in the efficacy 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?

It is not ethical to test the effectiveness of antidotes against toxic industrial chemicals (e.g. cyanide) in human clinical trials. Therefore, to demonstrate this, animal models must be used.

Supporting the study design is both the amount of toxic chemical in the blood/body following challenge and the amounts of antidote in the blood/body following administration. Calculating that the amount of antidote is sufficient to protect against the lethal effects of the chemical exposure is critical.

Which non-animal alternatives did you consider for use in this project?

Non-animal studies have already been carried out with the antidotes and those data will be used to inform the appropriate use of the antidotes in the animal studies proposed in this license application.

Why were they not suitable?

The effectiveness of the antidotes to save lives can only be ethically determined in a living animal studies not in human clinical trials. The interaction of the antidotes and the toxic industrial chemicals have been studied in non-animal experiments, these data have informed the planned study but cannot predict the outcome in a living 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?

Animal numbers were estimated using data from studies published in peer reviewed journals, those studies investigated the antidotes or toxic industrial chemicals (e.g. cyanide) that are proposed for use in this project under different experimental conditions. Data from those studies have been used to inform the selection of doses of both antidote and the toxic industrial chemicals that will be investigated on this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3Rs Experimental Design Assistant was used to plan the studies. Data from studies published in peer reviewed journals for the antidotes and the toxic industrial chemicals were utilised to indicate the likely outcomes and data from the proposed project. Unpublished proprietary data for the antidotes, obtained with agreement from other research groups, has also been used to inform the study design and animal numbers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be used to determine the appropriate dose of toxic industrial chemicals used as the challenge against which the effectiveness of the antidote will be demonstrated. Computer modelling and simulation of published data, unpublished proprietary data and data from experiments in this project will be used to predict the outcome of subsequent experiments in this project.

A pharmacokinetic model was fitted to concentration-time data determined from pigs following intramuscular administration of cobinamide. Unpublished data was received from collaborators and has enabled *in silico* simulation of intravenous administration. Although not verified against real intravenous data, the predicted concentrations from that modelling enable an informed approach to dose selection for efficacy studies, such that initial efficacy studies are able to proceed without prior *invivo* pharmacokinetics for cobinamide on this licence. Additional stoichiometric dose calculations, together with the predicted data from the simulations add to the confidence that the calculated dose will be efficacious.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 animals on this project (74%) will be terminally anaesthetised pigs. The first procedure they will undergo will be induction of anaesthesia and they will remain anaesthetised throughout the rest of the experimental protocol. The pain, suffering, distress or lasting harm will be minimised for these pigs as they will be unconscious.

The remaining animals (26%) will be pigs that are anaesthetised, then surgically prepared with cannulae in their blood vessels, to enable administration of antidotes and collection of blood samples. These animals will wake up from the anaesthesia and have a post-operative recovery period before antidotes are administered to them. Anaesthesia will prevent any awareness of the surgical procedures. Pain relief will be used before and after surgery to reduce pain, suffering and distress during post- operative recovery from surgery. The administration of the antidote is likely to cause sub-threshold or mild pain, suffering and distress to the pigs. The implantation of cannulae is being done to reduce the necessity for repeated needle punctures into blood vessels for the collection of blood samples, a method that will cause less pain, suffering and distress.

Why can’t you use animals that are less sentient?

The majority of the pigs used on the project will be terminally anaesthetised (non-recovery). For the pigs that will be conscious for the administration of antidotes and collection of blood samples, the absorption of those antidotes are likely to be reduced in anaesthetised animals. Thus study of that absorption is more appropriate in conscious animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The planned procedures will monitor the physiology of the terminally anaesthetised animals during the experiments, collecting many parameters from each animal to inform the outcomes of the study and to ensure the welfare of the animals is considered.

Behaviour of the conscious animals after administration of antidotes will be monitored for any adverse effects. Behavioural training will use positive reward to motivate the required behaviour (for example standing still for a sample to be collected), particularly during blood sample collection from cannulae.

Surgical implantation of cannulae will facilitate administration of the toxic industrial chemicals and antidotes and facilitate blood sampling whilst limiting damage to vessels through repeated access. Pain relief drugs will be given before and after surgery. Animals will be monitored as they recover from anaesthesia and will be regularly checked following surgery to ensure appropriate level of pain relief is administered and to monitor the progress of wound healing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs guidance as well as published guidance (Diehl *et al*, 2001) on the administration of substances to and withdrawal of samples from animals.

All surgery will be follow aseptic technique guidance (LASA: Education, Training and Ethics section; 2017 or subsequent updates to this guidance).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in the 3Rs are regularly communicated by our Named Information Officer. Any appropriate advances will be discussed with our veterinary staff and, where appropriate and compatible with the scientific aims of the project, these advances will be incorporated. I will actively maintain my CPD, attend relevant external scientific meetings and have meetings and teleconferences with international collaborators working in this area of research.

140. Roles of electrical activity in brain maturation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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, Development, Plasticity, Regeneration

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how ongoing changes in electrical activity can influence the development, plasticity and regeneration of neuronal circuits

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 provide novel basic knowledge about the development and maintenance of neuronal form and function in the mammalian brain. The new understanding we generate can be used as the basis for translational work aimed at reducing the burden of neurological disorders.

What outputs do you think you will see at the end of this project?

This is a basic neuroscience project, so its sole aim is to generate new data, knowledge

and understanding regarding the maturation, plasticity and function of neuronal networks. This novel information will be communicated in the form of peer-reviewed publications, as well as non-peer- reviewed preprints, conference presentations, and information disseminated online and on social media.

Who or what will benefit from these outputs, and how?

This is a project focused on fundamental, basic science: our outputs will add to knowledge of how the brain works, how it is built, and how it can change over time. Most immediately, our work will benefit the broader scientific community by providing new understanding and freely-available data which can be used by other scientists to inform, influence and integrate into their own investigations. Our research also has the immediate potential to benefit the public, by generating openly-available novel data on brain structure and function, and by public engagement in our discoveries.

In the longer term, we also hope that our work will prove to be of some clinically-related benefit. Although we focus purely on illuminating our basic understanding of the brain, the knowledge we generate could inform therapeutic approaches to repairing the diseased or injured brain. By focusing on the unique properties of newborn cells in old circuits, our work directly impacts on attempts to repair damaged or diseased brain tissue through the replacement of newly-generated neurons. And by investigating the ways in which neuronal plasticity can impact on the functional regeneration of sensory nerves, this project could influence attempts to encourage brain repair after insult or injury.

How will you look to maximise the outputs of this work?

My laboratory has a strong commitment to Open Science, so we look to maximise the outputs of our work by making our findings freely and publicly available to the widest possible audience at all times. This involves posting preprints of all manuscripts on the bioRxiv server, publishing our peer-reviewed work in Open Access journals (available from the time of publication), and depositing all of the raw data related to published papers in independent, freely accessible public data repositories (e.g. Dryad, Figshare, Neuromorpho.org). We also run a group website which has news and content specifically directed to the general public, and maintain an active lab twitter account to publicise our latest work.

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.

All animal experiments in this project will involve mice. Mice are the simplest available mammalian model with the appropriate neuronal populations, maturational phenomena and activity-dependent plasticity. Using mice will enable us to build on a large bank of existing knowledge, including specific data on olfactory system development and adult neurogenesis in this species. This will avoid unnecessary repetition, and will also allow us

to plan and perform the most appropriate experiments. Using mice also enables us to exploit existing genetic technology to label and modify activity in particular types of neuron, technology that amongst mammals is most advanced by far in this species.

Our main focus is on plasticity in the adult brain, so the majority of experiments in this project will take place in adult mice. However, we will continue to learn general lessons about neuronal maturation and plasticity by comparing early postnatal development with the maturation of immature neurons in the adult brain. For this reason, we will also perform experiments in young postnatal and juvenile mice.
We will not obtain any data directly from embryonic mice, but the project does require embryonic labelling approaches. These allow us to target specific neurons by accessing them at early prenatal developmental stages, before obtaining readouts from those neurons in postnatal or adult mice.

Typically, what will be done to an animal used in your project?

Although we will do some experiments on wild-type mice, most will involve transgenic animals (Protocol P1). Our most common approach will be to introduce additional genetic material in order to label or modify specific populations of neurons; this will be achieved by activating conditional gene expression with drug compounds (P2-7) and/or by surgical injection of viral vectors in specific regions at specific points in development (P3, P6-7). Many experiments will characterise the baseline process of anatomical and functional maturation in these specifically-targeted neuronal populations. However, in at least half of our experiments we will also employ manipulations to study the role(s) of electrical activity in shaping this maturational process (optional components of P5-7).
These manipulations include altering sensory experience by inserting a plastic plug into one nostril, directly changing the electrical activity of particular cells with drugs or by using flashes or light delivered via a surgically-implanted light guide, and/or inducing naturally-occurring nerve regeneration using a specific drug which is usually injected just once into the animal's abdomen. After these targeting and manipulation protocols, the majority of data collected in the project will actually be obtained from ex vivo tissue. However, in some cases we will use in vivo microscopy and/or electrical recordings (P7) to study the structure and function of neurons as they mature in the intact brain.

What are the expected impacts and/or adverse effects for the animals during your project?

The experiments in this project will not induce any deliberate or permanent adverse effects on the mice involved. Genetic manipulations will be used to label or modify a small fraction of neurons in the body, and are not expected to produce any behavioural effects. Manipulations of neuronal activity by altering sensory experience, or by inducing neuronal degeneration followed by regeneration, will themselves be minimally invasive. The degeneration they produce will affect olfactory sensory ability for at most several weeks before full regeneration occurs, and will not produce overt behavioural changes. Surgical procedures will not induce any significant lasting harm, and will be associated with rapid post-operative recovery. Most animals will undergo a single surgical procedure, and many animals (wild-type controls used for the description of normal maturational processes) will only be included in the licence when they are humanely killed for tissue harvesting by a non-Schedule 1 method. When it is experimentally necessary some animals will undergo multiple procedures over the course of several months of adult life, always with sufficient intervening time for full recovery. There is a low risk of complications associated with these surgical procedures, but the incidence and severity of such complications will be

minimised by adherence to best practice, close monitoring, and strict application of 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)?

Most procedures will be mild, involving transgenic breeding, non-surgical delivery of agents, or killing under terminal anaesthesia. All other, surgical procedures will be of moderate severity, although the majority of these will be minimally invasive and involve placement or removal of nasal plugs under brief gaseous anaesthesia.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are no alternatives to using animals in the current project. Our aim is to describe the activity- dependent mechanisms that shape brain maturation and function. This is crucial basic information which is currently unknown, and which – because of its dependence on sensory information and complex neuronal circuits – needs to be studied in the intact brain in vivo. Moreover, studying neuronal plasticity requires precise targeting of specific cell types and the ability to manipulate genetic, cellular and environmental factors, invasive approaches which are not possible in human subjects.

Which non-animal alternatives did you consider for use in this project?

We considered a number of non-animal alternatives for use in this project, and will continue to do so going forward. These include ex vivo human tissue samples, cell lines generated from human samples (hIPSCs), and computational in silico models.
We also actively considered partial replacement approaches, including non-regulated model organisms and cell cultures prepared following Schedule 1 killing. Indeed, this project is preceded, informed and complemented by a significant amount of ex vivo work of this kind. Our laboratory works extensively on dissociated neuronal cultures, and the animal research proposed in the current application has been led by results generated by these in vitro studies. Throughout this project we will use ex vivo cultures to plan the most effective experiments to perform in vivo, and where appropriate to provide effect size and variability estimates to allow power analyses for minimal group size calculations.

Why were they not suitable?

In most cases non-animal alternatives are simply unavailable. Using the EURL ECVAM Search Guide, precisely-defined searches for relevant terms (“olfactory”, “neuronal

plasticity", "adult neurogenesis", "nerve regeneration") produced no results in 3Rs added-value databases (NC3Rs Gateway, DB-ALM). Searches within the broader PubMed database found no hits for studying olfactory bulb function in post- operative human brain tissue, and no available in vitro cell lines for olfactory bulb hIPSCs. Non- regulated organisms such as Drosophila lack fundamental and crucial features of the olfactory-processing networks that form the basis of this project.

In silico models are available for some basic properties of olfactory bulb networks, and remain a strong area of focus in the field. However, no current computational models replicate the full complexity of different cell types (and subtypes) within bulbar circuits, let alone the impact of environmental alterations or nerve regeneration on those networks. We will continue to study these exact processes in mouse models, with the aim of producing accurate mathematical models of neuronal plasticity and development 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?

This is a project investigating novel, basic and fundamental features of brain function, which uses a wide variety of techniques to address a series of individual scientific sub-questions. For these reasons, while it might prove possible to use power calculations to estimate the numbers of animals required for a given individual experiment (in those cases where good published or pilot data are available), those calculations cannot necessarily be applied to all of the experiments planned in this project, nor can we accurately predict exactly how many of each experiment will take place over 5 years. Unpredictable future variations in funding support will also impact on the number of experiments we are able to perform. Our estimates of animal numbers are therefore justifiably imprecise.

Nevertheless, we can form a reasonable estimate of the total numbers of animals used by drawing on our considerable expertise in performing these kinds of studies. Our group has been investigating the impact of electrical activity on brain maturation for over a decade, and has extensive experience in all of the protocols and approaches detailed in this project. We can make good estimates of a) the likely effect size of individual comparisons, b) the average number of data points (e.g. cells recorded, or cells imaged) collected per animal using a given technique, c) the proportion of failed approaches, either because of unforeseen experimental error, rare surgical complications, or sheer biological unpredictability, d) the efficiency of breeding particular transgenic lines and crosses, and e) the total number of experiments likely performed by a research team of our size over 5 years. We have also been informed by the animal numbers listed on our previous project licence, in comparison with the carefully recorded and reported numbers of animals we actually used. Together, these factors have combined to produce the estimates of animal numbers we have listed for each protocol.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A number of experimental design measures ensure the minimum number of animals will be used in this project. Overall, good experimental design principles – use of appropriate controls, randomisation and blinding – are used throughout to ensure experimental reliability and validity, and to minimise experimenter-induced variation. This will allow us to detect given effect sizes with lower animal numbers. Specific design features include the use of paired comparisons where possible (for example, the within-animal chronic imaging/recording experiments in Protocol P7); this will reduce animal-to- animal variability and reduce the total of animals used.

We also use power analysis (with e.g. GPower, or calculated with custom-written scripts) based on previous data, published data and/or pilot in vitro data where possible. This allows us to minimise animal numbers for an experiment whilst ensuring we generate enough data to detect appropriate effect sizes. We also use state-of-the-art statistical analyses of our experimental data to ensure that maximum power is obtained from the minimum number of mice. For example, we will continue our recent use of multi-level statistical approaches which allow multiple measures to be obtained from individual animals without violating assumptions of statistical independence.

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 transgenic breeding as efficient as possible, using existing expertise as well as guidance from the NC3Rs and Jackson Laboratories on best practice. This will be facilitated by the fully electronic mouse record keeping system recently introduced to our animal facility, which will also ensure no unnecessary duplication of transgenic lines that are available elsewhere at our institution.
Using both sexes for all experiments will also minimise mouse numbers, as will high quality animal welfare to minimise suffering and distress, and therefore reduce inter-animal variability. This includes refinement of surgical techniques where possible (see below).

We will also reduce numbers by extensive use of cell culture work to inform experimental design and, where appropriate, provide pilot data. Other use of parallel approaches will reduce numbers further – for example, collecting multiple types of information from individual animals by obtaining tissue for further anatomical or molecular analysis after live functional data have already been obtained from the same mouse. We will also keep such tissue securely and under safe record (a lab ‘brain bank’) for use in appropriate 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.

The animal experiments in this project will use mice. Because mice are the simplest, least

sentient available model with the appropriate activity-dependent neuronal plasticity and maturational phenomena, as well as possessing the particular types of neurons and circuits on which we focus, using them causes the least possible animal suffering.

Our research goals require the targeting of specific types of neuron and the alteration of electrical activity in brain tissue, in animals of different ages. The methods outlined in the protocols below have been carefully chosen to fit these objectives while producing the minimal possible animal suffering.
Some have been expressly chosen over more common or conventional techniques because they provide substantial refinement: naris occlusion by nasal plug (optional components of Protocols P5-P7) instead of the far more common but significantly more painful cauterisation approach, for example.

Movement through the protocols and continued use has been designed such that individual animals will only rarely undergo more than one major (>10min) surgical procedure. Cannula/light guide implantation for in vivo light stimulation, for instance, will wherever possible be carried out during the same surgical anaesthesia employed for adult brain injection. Moreover, the vast majority of animals will undergo a single procedure, and many animals (wild-type controls used for the description of normal maturational processes) will only be included in the licence when they are humanely killed for tissue harvesting by a non-Schedule 1 method. And, although chronic in vivo imaging/recording experiments in protocol P6 necessitate multiple procedures in individual animals, this longitudinal design will substantially reduce the number of animals used (see Reduction). Of course, good surgical technique, animal husbandry and veterinary advice will ensure that all procedures cause the minimal possible suffering. We do not propose any protocols with substantial severity.

Why can’t you use animals that are less sentient?

This project focuses on plasticity in the postnatal brain, especially in adults. Using more immature life stages would therefore not be appropriate for our scientific objectives, although some embryonic procedures will be used to target specific cell types for later postnatal characterisation (P2-3).

Less sentient species would not allow us to address our scientific objectives either. Invertebrate models simply do not have many of the key features we will study in this project. Drosophila, for example, lack local dopaminergic neurons in their version of the olfactory bulb, do not have adult neurogenesis of specific interneuron populations, and display many significant differences in basic cellular properties related to neuronal polarity. Other less sentient potential vertebrate models such as zebrafish have a very different basis for their sense of smell (aquatic vs airborne odorants), and the functional roles of dopaminergic neurons in the zebrafish olfactory bulb are known to be qualitatively different to those in mammals.

Our choice of procedures always uses less sentient states wherever possible. We use terminal anaesthesia for many in vivo experiments (P7), the majority of our data comes from ex vivo readouts following killing under terminal anaesthesia (P4-6), and we complement our in vivo work with dissociated culture approaches wherever we can.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our animals will be monitored closely using both quantitative and qualitative indicators by experienced, trained staff. Responding to the outcomes of such careful monitoring can involve better post-operative care implemented directly, or after discussion with the NACWO and/or NVS. Recent examples of this include changes to reversible injectable anaesthetic, and the use of more easily accessible food and water in the immediate post-operative period.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As well as receiving regular N3CRs updates, we will monitor the published literature for specific refinements that might be possible in particular experiments. This will be aided by automatic search updates from Google and PubMed, and automatic Table of Contents updates from relevant journals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will have regular informal and formal meetings with the NACWO and NVS to discuss the potential implementation of recent advances. I have registered with the NC3Rs website for regular email and newsletter updates. We will also continue to closely follow the peer-reviewed literature as well as preprints and social media, including the latest applications in biostatistics. If any of these avenues suggest alternative protocol steps, we will apply for PPL amendments as appropriate; otherwise novel approaches will be implemented as soon as practicable.

141. Drug Discovery & Pharmacokinetics of Small Molecule Cancer Therapeutics

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

drug discovery, cancer, pharmacokinetics, novel treatments, 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?

Studies carried out under this licence will be used to support determination of the in-vivo pharmacology and efficacy of novel treatments for a range of different cancers, and to offer the opportunity for these treatments to better aid the understanding of the underlying biological processes involved. More specifically, these studies will be used in drug discovery projects, where small drug molecules are administered to mice to investigate preliminary in-vivo pharmacokinetics (PK) and efficacy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Drug Discovery programmes of research are used to advance the development of new and more effective treatments for cancer, with less associated side-effects. Of particular note is the objective to generate novel treatments that address areas of high unmet need, including gaining a better understanding of processes such as metastasis that underlie

extremely poor prognosis in cancers such as prostate, breast and brain.

In parallel, the high quality compounds generated and evaluated by the Drug Discovery programmes will allow researchers to better unravel the complexities of cancer, and identify new and more effective therapeutic opportunities.

The studies covered under this PPL will ensure the most appropriate dosing and assessment of compounds in future efficacy studies in mouse models of cancer. This ensures that future in-vivo studies are done with the minimum number of animals and achieve the clearest possible outcomes.

What outputs do you think you will see at the end of this project?

The outputs will be data demonstrating the characteristics of adsorption, distribution, metabolism and excretion (pharmacokinetics) of the test compounds in animal models, providing an early indication of their potential effectiveness as novel therapies. The data from these initial pharmacokinetic studies will provide valuable information on appropriate future dosing regimes and any adverse reaction an early indication of potential drug toxicity. Follow on efficacy studies will generate new information on the underlying biology of the particular cancer target being studied, by providing the opportunity for the collection of research samples and the measurement of relevant biomarkers by research support laboratories, as an indication of drug effectiveness (efficacy).

These data may lead to scientific publications, generate Intellectual Property and enable Go/No Go decisions on whether to progress promising compounds into clinical trials in patients.

Who or what will benefit from these outputs, and how?

The Drug Discovery Programme is expected to advance the development of new and more effective treatments for cancer with less side effects. Of particular note is the objective to generate novel treatments that address areas of high unmet need including processes of invasion and metastasis that underlie extremely poor prognosis for some cancers. In parallel, the high quality compounds generated and evaluated by the Drug Discovery Programme will allow researchers to better unravel the complexities of various target cancers and identify new and more effective therapeutic opportunities.

The studies covered under this PPL will ensure the most appropriate dosing and assessment of compounds in future efficacy studies in mouse models of cancer. This helps to ensure that future studies are done with the minimum of animals and achieve the clearest possible results.

How will you look to maximise the outputs of this work?

A significant benefit of this animal project licence is to establish a center of excellence for early in-vivo drug screening giving researchers access to world-class scientific expertise on novel cancer therapies and unpublished data regarding the specific compounds being investigated. Close communication between the laboratory, offering this service, and research groups is likely to maximize outputs from the animal experiments as well as speeding up the development of new therapies and contributing directly to new literature publications.

Species and numbers of animals expected to be used

- Mice: 3880

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 the species with the lowest neurological sensitivity likely to produce data predictive of drug effect in man.

Mouse models are among the most valuable tools in cancer research and researchers use them for many types of studies, from identifying possible new cancer treatments to finding new clues about cancer biology. Mouse tumour models are initially created by implanting a fragment of a human tumor into a mouse. Studies have shown that the resulting mouse model largely retains the genetics of the human tumors from which they were initially created. This is important, because results from drug efficacy studies are increasingly being used to decide whether to move experimental drug treatments forward into human clinical trials.

Although there can be differences in drug metabolism between species, studies have shown a good general agreement of pharmacokinetics between different mouse strains, and gives valuable preliminary information of drug behavior in humans.

Typically, what will be done to an animal used in your project?

In preliminary experiments, where the characteristic of the new drug molecule is being investigated, mice will be dosed with test compound for up to 4 weeks prior to termination. The dosing volumes used will not exceed those suggested for mice in local Institute and other published guidelines (Workman et al., Guidelines for the welfare and use of animals in cancer research, British Journal of Cancer (2010) 102, 1555-1577). The frequency of dosing will depend on the cancer being investigated and the blood concentrations of the compound. The commonly used routes of administration will include injection into the abdomen, injection into a vein and oral dosing.

Once the behaviour of the compound has been characterised, efficacy experiments will include where some animals receive donor tumour tissue by subcutaneous injections or small surgical insertion. Once the tumour has grown to a sufficient size, animals will receive the test compound and undergo blood sampling. The majority of mice will experience only mild or moderate suffering, with repeat injection/administration of the compound. Exposure to the agent will be typically daily for 7 days, but could be extended up to a maximum of 4 weeks, at which point the animals will be killed. Mice will be regularly weighed to ensure that they aren't subject to significant weight loss or other clinical signs.

Some experiments may include temporary separate caging of mice, to allow the collection and analysis of urine from individual animals, but this separation of animals would be limited to no longer than 24 hours at any one time.

What are the expected impacts and/or adverse effects for the animals during your project?

The administration of test compounds should result in no more than transient discomfort and no lasting harm. Occasional unexpected adverse effects may occur because the mice will be exposed to acute doses of the test compound for the first time. Acute effects could include allergic reaction, local tissue damage due to direct contact with the agents, or abdominal pain (after intraperitoneal dosing). These adverse effects will likely manifest themselves within the first 2 hours of dosing and correlate with the highest concentrations of drug in the bloodstream. Chronic adverse effects from repeat dosing may include gut toxicity (demonstrated by diarrhoea or intestinal impaction), and bone marrow toxicity. The duration of any adverse effects will be monitored closely and, based on the clinical signs, animals will be killed to minimise any pain and suffering if these effects are not improving or worsening.

In efficacy studies, the implantation of tumour cells should not create side effects other than a potentially mild immune response. The growth of subcutaneous tumours will be monitored regularly and not allowed to grow sufficiently large to impede normal animal behavior or exceed limits published in the literature on animal welfare (Workman et al.).

Expected severity categories and the 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 severities in this licence are predicted from experience of other similar licenced protocols at the establishment Protocols 1 & 2, where novel compounds are administered to non-tumour bearing mice, the predicted severities are 74% mild and 26% moderate. For protocol 3, where novel compounds are administered to tumour-bearing mice, the predicted severity is 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?

It is only in whole animals that the complexity and interplay between biological systems can demonstrate if a compound will be absorbed after dosing and have an effect on biological processes of cancer that are expected to translate into clinical effectiveness.

Which non-animal alternatives did you consider for use in this project?

In-vitro testing will be used to extensively evaluate the potential of compounds in terms of efficacy, toxicity and drug properties prior to compounds being selected for testing in animal models, and will include other sources of ex-vivo information such as in-silico

modelling of drug properties. This preliminary ex-vivo information must exist before testing in animals is considered.

Why were they not suitable?

Testing the compounds in animal models better reflects the potential for clinical efficacy. In addition, the pharmacokinetics of compounds, which is critical for determining the exposure of the target to the molecule, cannot be predicted in-vitro. This requires a mammalian system and exposure of the compound to complex processes such as adsorption, distribution, metabolism and excretion, which can be evaluated by the analysis of post-administration blood sample concentrations over a time-course.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Protocol 1:

For preliminary PK studies the study design will usually include two compound dosed groups of 21 mice (e.g. iv & po routes of administration), with 3 terminally bled mice per timepoint and seven timepoints across the study, allowing the determination of the half-life of the drug plasma concentration. Thus, a total of 42 dosed mice and 3 control mice administered the formulation vehicle only will be used per study. It is anticipated that the laboratory will support no more than one Protocol 1 study per month across the 5 year period of the licence, i.e. a maximum total of 2700 mice.

Protocol 2:

In dose setting studies, the experimental design may be more varied than Protocol 1 and based on the planned number of dose escalations and duration of the repeat doses. However, for the basis of calculating animals numbers three dose groups of 21 animals have been used, with 3 terminally bled mice per timepoint and seven timepoints across the study (daily blood sampling to determine the steady-state exposure over a one week period). For each dose group, a pilot dose escalation administration will be used (n=2 mice) to monitor for adverse effects and a control group of 3 mice dosed with formulation vehicle alone. Therefore a total of 78 mice will be used per dose setting study. Demand for dose setting studies (Protocol 2) is anticipated to be lower than Protocol 1, with no more than two studies performed per year across the 5 years of the licence - thus a maximum number of 780 mice.

Protocol 3:

For each efficacy study that will be performed, the number of mice per group will be determined by power calculations based on expected up or down regulation of the biomarker response after treatment and the analytical variability in measuring the biomarker (e.g. tumour size). From previous experience of performing preliminary efficacy

studies in the establishment, involving a single compound treatment at a single dose level, n=10 per animal group has typically sufficed for monitoring the effect of agents in most animal cancer models. With a typical study including three dosed groups (at three different dose levels or dose combinations with other drugs) and one control group, it is estimated that a total of 40 mice will be used per study. With all dose setting studies followed by an efficacy study (i.e. two efficacy studies performed per year). It is estimated that a maximum total of 400 tumour bearing mice will be used over the lifetime of the project protocol.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where there is preexisting experimental data, software (PC Modfit version 6.0 or Phoenix WinNonLin) will be used to simulate the in-silico pharmacokinetics of the test compound in the animal model. This will assist in the optimisation of dosing regimes and sampling protocols in order to maximise the amount of data from the minimum number of animals. For each new project there will be an experimental design meeting with a statistician to ensure the optimum number of animals are used to meet the objectives of the study.

The application of micro-sampling techniques will also be considered on a project by project basis, where blood concentrations are sufficiently high, to enable the use of serial sampling of animals and 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?

The laboratory will use state-of-the art, high sensitivity instrumentation to measure drug concentrations in blood plasma samples. High sensitivity of detection has the potential to enable the use of small sample volumes and the application of micro-sampling techniques, leading to the potential for serial sampling and a reduction in animal numbers required to generate data for pharmacokinetic analysis, where this is practical. Furthermore, the laboratory will employ the use of robotics for the preparation of samples, where practicable, which will reduce experimental data variability for significance testing 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.

This licence uses wildtype and immunocompromised mice. It is essential that immunocompromised mice are used in efficacy studies in order for non-mouse cell lines to grow tumours successfully without rejection by the host. A subcutaneous tumour is the least stressful for the animal for the purpose of assessing drug efficacy.

The least harmful route of administration will be used for dosing which is compatible with

the compound properties, e.g solubility. Any solubility issues will have been previously identified during in-vitro (unregulated stage) of the compound development.

Why can't you use animals that are less sentient?

Mice are the species with the lowest neurological sensitivity likely to produce pharmacokinetic data predictive of translation into humans. Also, the majority of animal models of cancer used in efficacy studies have been developed in mice, and there is a broad base of literature on cancer biology and pharmacology in mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animal facility at the research establishment is well established with a depth of experience in refinement of conducting preclinical studies and general animal husbandry. The procedures that will be used under this license are well-practiced and standard within the establishment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The animal facility adheres to best practice guidance, currently the "NCRI Guidelines for the welfare and use of animals in cancer research" by P.Workman, et al., Br. J. Cancer (2010) 102, 1555-1577 .

For surgical procedures:

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/

For recording and reporting on experiments:

The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, Nathalie Percie du Sert et al., PLOS Biology, July 14, 2020. https://doi.org/10.1371/journal.pbio.3000410

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Animal Welfare and Ethical Review Body at the research facility disseminates information, guidance and best practice to staff involved in animal experiments. The project licence holder will stay informed of advances in the 3Rs through discussion with colleagues and named persons including Named Veterinary Surgeon (NVS) Named Animal Care and Welfare Officer (NACWO) and direct membership of the AWERB committee at the research facility.

142. Investigation of genes predicted to be involved in blood cell production

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Blood cells, Human, Genes

Animal types	Life stages
Zebra fish	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate and characterize genes that are predicted to be involved in the development of blood cells in health and disease, with the intention of identifying genes that are important in human haematological (blood) 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?

Blood cells are produced by the bone marrow and contribute to several essential functions in the body. Red blood cells are vital for carrying oxygen in the blood, white blood cells are an important part of the immune system, and platelets contribute to the body's ability to form blood clots and prevent excessive bleeding. Haematological disorders (blood disorders) are a common cause of poor health in the UK and globally and include numerous diseases ranging from the very common to the very rare. For example, 15% of women of childbearing age and 11% of children under 5 years living in the UK have some form of anaemia (WHO data, 2016), while there are approximately 15,000 people living with sickle cell disease in the UK (Sickle Cell Society data, personal communication), and

less than 3,000 individuals with severe inherited thrombocytopenia (too few platelets) in the UK (UKHCDO Bleeding Disorder Statistics 2017/18). What unifies all these conditions is an abnormality in the way that blood cells are produced in the bone marrow. It is becoming increasingly apparent that genetic regulation of blood cells is important in all these conditions - directly, in the inherited disorders that may be passed from one generation to the next, and more subtly, in those acquired conditions where genetic differences between individuals makes them more or less susceptible to developing a blood disorder.

Our research focuses on the identification of new genes that are important for regulation of blood cell development and function. The group uses clinical and next generation sequencing data from patients with inherited blood disorders and healthy individuals alongside more basic laboratory science research techniques to achieve this. Identification of the genes involved in the regulation of blood cell production is important for several reasons. Firstly, it provides new candidate genes that might be directly involved in inherited forms of blood disorder. Secondly, it will help explain why some people are more prone to developing acquired blood disorders (e.g. due to nutritional deficiencies or cancer treatments) than others. Thirdly, identifying genes that are involved in blood cell production may provide future treatment targets.

In order to reap these longer-term rewards, we need to be able test whether the genes we identify through clinical and research sequencing of human genomes are really involved in blood cell production, or whether we have found a chance association. One way of approaching this is to use zebrafish to look at the effect of altering the activity of genes of interest. This approach can be used alongside other non-animal research techniques to try to understand the role of a gene in the regulation of blood cell production. It is important to correctly identify genes that are involved in blood cell production, before moving on to applying these results to human diseases and their treatment.

What outputs do you think you will see at the end of this project?

The data generated in this project is expected to identify and characterise new genes involved in the regulation of blood cell production, and to improve bioinformatic and in silico approaches to candidate gene identification. I expect this new information to be shared with the scientific and medical communities through publication in haematology and genetics journals and through presentations at national and international conferences. In order to increase the dissemination and accessibility of these outputs, high quality open access journals will be prioritised.

It is possible that a gene or genes may be identified that are involved in a new form of human inherited blood cell disorder. This may lead to changes in the genetic testing offered to patients with inherited blood cell disorders through the NHS and other health care systems and should result in more specific information being available to patients about their condition.

Who or what will benefit from these outputs, and how?

In the short term, identification of new candidate genes involved in human blood cell development will be of benefit to the scientific community, in providing new hypotheses for testing or candidate biological pathways for manipulation by drugs or other methods. This impact is expected to be seen within the first 1-3 years of the project but will continue throughout the life of the project.

In the medium term, patients with inherited blood cell disorders are expected to benefit, as the identification of new genes involved in blood cell development should result in some of these patients obtaining a precise genetic diagnosis. This is expected to result in more personalized information about prognosis and treatment options and is anticipated within 3-5 years of the start of the project. Over the same time course, I anticipate that pharmaceutical companies and biotechnology companies will benefit from the identification of novel targets for therapeutic and diagnostic interventions.

In the long term, the NHS and other healthcare systems are likely to benefit from improved diagnostic rates in patients with inherited blood cell disorders, and more therapeutic options available to them. Patients with acquired blood cell abnormalities, such as due to drug treatments for other conditions, are also likely to benefit from improved therapies that will result from a better understanding of blood cell development. This impact is expected beyond the end of the project (5+ years).

How will you look to maximise the outputs of this work?

Collaborative work is crucial for this project. I have collaborations with other research groups in the UK and overseas, with particularly strong links with blood cell groups in Europe and North America. This helps to reduce the risk of unknowing duplication of experiments by groups working in a similar research field.

New knowledge will be disseminated in high quality, open access peer-reviewed journals and at national and international conferences. It will also be shared via online pre-publication archives, with the aim of reducing unnecessary duplication.

Where possible, unsuccessful approaches and negative results will be published in the scientific literature. This information will also be discussed at relevant national and international conferences and collaborative meetings.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 3,500 adult fish

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 of the species suitable for the study. The genetic tractability of zebrafish facilitates the manipulation needed to undertake the work and their translucent bodies enables live imaging of blood cells to be undertaken in the intact animal, which would not be possible in other species, such as mice.

The majority of work is non-regulated and involve studying zebrafish embryos prior to 5 days of age, at which point their nervous system is insufficiently developed to enable them to experience suffer. A small number of fish per gene will be grown to maturity to ensure that any age-related effects are detected, and to allow for breeding.

Typically, what will be done to an animal used in your project?

On arrival in the aquarium, purpose-bred fish for investigation of blood cell disorders will be given an appropriate amount of time to settle in to their environment before breeding is commenced. The resulting fish eggs will be collected and injected with a small amount of liquid that will target the gene of interest and silence it. The eggs will be allowed to develop into fish embryos in an incubator. The majority of research will be carried out on embryos or lava stages of the fish before 5 days of age . This would include checking that the gene has been properly silenced and monitoring the blood cells develop by imaging with a microscope, before killed the embryo to enable tissues to be collected for further analysis.

A small number of fish embryos, in which the relevant gene had been silenced, will be grown to beyond 5 days of age. These fish will undergo be anaesthatised to allow for genotyping, imaging by microscopy and blood sampling prior to killed by anaesthetic overdose. After they had been killed, samples of other tissues will be taken for further analysis. A small number of fish in which the relevant gene had been silenced may be used for breeding, if the gene is thought to be strongly implicated in blood cell development.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of fish are not expected to suffer any adverse effects as a result of the procedures undertaken.

A small proportion of fish may have deficiencies in blood cells that could result in reduced vigour (due to anaemia), or to have an increased risk of bleeding or infection, or to have impaired hearing or balance. Although persistant, these changes are not expect to result in any alteration in the aprearance or behaviour of the fish.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity for this study is mild, however, the suffering incurred by the majority of fish is expected to fall below threshold (estimated at >80%). The remaining no more than 20% may experience mild suffering as described above.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Unfortunately, in vitro systems cannot model the 3D environment of haemopoietic tissue and the complex relationships between stem cells, developing blood cells and support cells. Furthermore, genes that are involved in the regulation of blood cell development may

also have effects in other organs - this is seen clearly in several human genetic disorders in which abnormalities of the blood are seen alongside abnormalities of other organs including (but not limited to) the kidneys, eyes, ears and brain. The use of animals in this project is essential in order to enable the assessment of other organs that might also be affected by changes in the genes under investigation.

Which non-animal alternatives did you consider for use in this project?

We have considered use of computational or in silico methods for determining whether variants identified in particular genes are likely to have functional effects on how the protein functions, and predict from that the likelihood of those variants causing disease.

We also considered work on human blood and bone marrow biopsy samples, and blood cells grown from donated stem cells. However, these approaches have their limitations. The genetic variation seen between humans makes interpretation of individual genetic changes very difficult. Culture or co-culture of haematopoietic cells alongside stromal cells does not completely replicate the normal process of haemopoiesis.

We will use in silico and in vitro approaches alongside our zebrafish work, to allow us both to reduce the number of experiments requiring animals and to reduce the numbers of animals used in each experiment. In vivo experiments in adult animals are only used where the specific objective cannot be achieved by using either non-animal methods or using unregulated larval stages of zebrafish development.

Why were they not suitable?

These non-regulated approaches were not adequate on their own to address the research questions posed. This does not mean that they cannot be used to reduce the use of animals, but they cannot fully replace the use of the zebrafish model in addressing the aims of the study.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 requested is based upon an estimate of the likely number of genes and cell types that will require manipulation in order to meet the stated objectives. In estimating the group size for the studies I have considered my own previous experience as well as data published by others. Due to the uncertainty of which genes will be investigated over the five years, it is hard to predict exactly how many adult fish will be required - for example, if the effects of gene silencing are more subtle than expected, or if more genes of interest are identified, then the number of fish required will also increase. The vast majority of animals requested will not be used in experimental studies but are required in order to breed animals with the genetic make-up needed for the experimental study. Here I have further drawn on the experience of other zebrafish groups working in the same aquatic facility, as similar husbandry approaches are likely to be relevant. For

example, for generation of a CRISPR frameshift mutant, local experience suggests that at least 3 tanks of injected fish would need to be raised, and that for homologous recombination, 2-3 times more might be needed.

I have therefore estimated my requirements as follows:

To breed and maintain stocks of fish:

Maintenance of two wild type lines, 2 tanks of each, exchanging at least every 1.5 years to maintain the health of the fish:

2 x 2 = 4 tanks, x 16 fish per tank x 3.4 (for exchange) = 218 fish.

Maintenance of six transgenic lines, 2 tanks of each, exchanging every 1.5 years: 6 x 2 = 12 tanks, x 16 fish per tank x 3.4 = 653 fish.
Maintenance of 10 mutant lines, 2 tanks of each, exchanging every 1.5 years:
10 x 2 = 20 tanks, x16 fish per tank x 3.4 = 1,088 fish.

Total = 1,959 fish, rounded to 2,000 for the licence application. To generate genetically altered fish:
10 new mutants made using CRISPR, requiring 4 tanks each. 40 tanks x 16 fish = 640 fish.
2 mutants actually requiring homologous recombination x 8 tanks each. 16 tanks x 16 fish = 256 fish.
Total = 896 fish, rounded to 1,000 for the licence application. To phenotype the wildtype and genetically altered fish:
This estimate is harder to come to with accuracy as the nature of the genes / mutants is as yet unknown. However, 500 fish would allow 50 fish per mutant line for protocol 3 (assuming 10 mutants investigated). Group sizes of 25 fish would be unlikely to be required, certainly not for all 10 lines being investigated, and where possible experiments will always be planned to maximize the information obtained from wildtype fish. This estimate therefore should give me enough flexibility that I would not need to ask for an extension to the number of animals needed.

At each step I have rounded up in order that I feel confident that I can remain within the limits of the project licence whilst still answering the scientific question and dealing with unexpected technical or biological issues.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The group size was estimated on the basis of previously published data investigating aspects of blood cell development in zebrafish. I will use the Experimental Design Assistant (EDA) from NC3R to plan all experiments and recognize that pilot experiments will be important to refine my group 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?

A pilot study using wildtype zebrafish embryos prior to 5 days of age will be undertaken to optimise certain techniques that are not dependent on using transgenic embryos and will further inform power calculations for individual sections of the project.

Bioinformatic and in silico techniques will be used to guide candidate gene selection and inform the experimental design. This ensures that only genes with a high prior probability of being relevant to blood cell development are identified and enables the analysis to focus on the biological pathways most likely to be involved. Where appropriate, experimental cell lines will also be used to supplement the in vivo work performed in zebrafish.

The vast majority of fish used are required for breeding. Care will be taken to ensure that the breeding programme is undertaken with optimal efficiency and that where possible it is co-ordinated with any other groups that require fish with the same genetic background.

To reduce the number of zebrafish that need to be grown to maturity, I will ensure that histological specimens and control tissues are shared with other research groups. When adult fish are killed at the end of the protocol, I will ensure that they are used fully and that where appropriate, samples are taken for storage to avoid the need to duplicate the experiment again 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.

Zebrafish are the least sensitivity of the species suitable for study haemopoiesis, and therefore selection of this species minimises pain, suffering and distress. The vast majority of the planned research will be conducted on early stage zebrafish embryos (prior to 5 days of age), while they are still insufficiently developed to experience suffering. The use of more mature fish will be reduced as much as possible.

Why can’t you use animals that are less sentient?

The majority of data will be collected from larval stages of zebrafish development, however, to generate these still requires the use of adult fish for breeding. A small amount of data will be collected from mature animals, but these will predominantly utilise tissue samples collected after the the fish have been killed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be carefully carefully for any signs of suffering by highly experienced staff within the aquarium unit in addition to the researchers. All procedures that could cause the fish pain, suffering or distress will be conducted under general anaesthesia. Upon recovery from anaesthesia any fish that shows more than transient adverse sign will be humanely killed. Where appropriate, environmental enrichment approaches will be used to support the fish being used within the breeding protocols.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All procedures will be conducted in accordance with LASA and NC3Rs Guidelines for best practice, and zebrafish husbandry will be performed in line with FELASA recommendations.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There is a highly active zebrafish network in the institution, with regular journal clubs and work in progress meetings. These ensure researchers stay informed of 3Rs developments and provide opportunities to discuss the best ways of implementing these. Attendance at scientific conferences will ensure that researchers remain up to date with advances not only in the in vivo techniques, but also in additional non-animal techniques that may be able to refine or replace zebrafish work. Researchers will also stay abreast of advice and meetings specifically offered by the N3CR organisation and my local named persons.

143. Liver regeneration, repair 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

Cancer, Liver, Regeneration, Therapy, Genetic models

Animal types	Life stages
Mice	adult, pregnant, 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?

This project aims to provide a deeper understanding of how livers regenerate and grow abnormally in cancer. We will use animal models combined with our expertise in cell culture with the aim of translating basic science into human clinical trials in primary liver 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?

Primary liver cancer is the third most common cause of cancer related mortality worldwide. Its most common form, hepatocellular carcinoma (HCC), typically occurs on a background of chronic liver disease. Chronic liver disease itself is a major worldwide health burden also. Deaths from liver disease now account for the third cause of premature death in UK. Rates of both chronic liver disease and HCC continue to increase in the UK and most of the Western World.
Whilst the liver is capable of highly efficient regeneration in some patients, others with apparently equally severe disease in the early phase go onto develop a progressive liver syndrome characterised by a failure of regeneration, multi-organ failure and death. We remain with a limited understanding of this process and currently there are no specific therapies capable of improving liver regeneration.

Similarly, whilst the therapeutic options for HCC have come a long way in the last 20 years, this remains one of the cancers with the worst outcomes. Major strides have been made in our understanding of the drivers of liver cancer and how they might be targeted either singly or in combination, especially with immune modification. This offers the promise of revolutionising therapies and outcomes for these patients.

Studies from this project license are designed to provide new insights into the roles that specific cells populations play in liver regeneration and carcinogenesis, and how they are controlled within living mammals. This body of work aims to identify what is different between a cell which can and does regenerate the liver compared to those that cannot or do not and what critical drivers cancer cells rely on to survive. We also have a major focus on how the cancer cells interact within a complex cancer environment made up of many different types of cells and how they evade destruction by the immune system. These studies should identify potential key players in the signalling pathways which control liver regeneration and crucially do this in the setting of models of liver disease which are applicable to human patients. As such it forms part of a larger local, European/global drive to develop regenerative therapies both in the liver and other organ systems. We are integral to this effort, participating in major international consortia.

What outputs do you think you will see at the end of this project?

We will generate knowledge into how liver tumours form and grow and most importantly how they respond, or fail to respond, to specific forms of cancer therapy. These will include both drug therapy, surgical resection and radiotherapy, potentially in combination. We aim to understand how tolerable these therapies are both on their own and also in combination with other commonly used medicines (e.g. paracetamol) which may predispose to damage of healthy liver cells. We also aim to improve our understanding of how liver and other

tumours spread around the body.

Outputs will be in the form of new information and knowledge together with experience of which are the best preclinical models to use to study cancer. These will be disseminated by publications, presentations and through the media. A key output will be the translational of our findings into patients with cancer through establishing clinical trials; something we have already achieved.

Who or what will benefit from these outputs, and how?

In the first 1-3 years the key outputs will be publications and knowledge. These will also include public engagement through both written and audiovisual media. Additionally, in year 3-5 it is likely we will begin to see outputs from clinical trials in cancer patients with therapies used in the preclinical models.

How will you look to maximise the outputs of this work?

We collaborate widely both within our establishment and with other centers both nationally and internationally. We will share our model systems and the knowledge of how to use them optimally. We will where appropriate, share the complex model systems we use with other researchers. These studies will be disseminated to the broader scientific community and particularly to those in cancer/regenerative cell biology and liver disease by publishing in high profile academic journals and presentation at scientific and clinical meetings. We will also be active in public/patient involvement.

Species and numbers of animals expected to be used

- Mice: 29900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 they represent a highly controlled model system which has an intact immune system and is relevant to human and other mammals. These models are particularly powerful due to the wide array of model systems (e.g. ability to activate cancer genes and relevant experimental techniques to scientifically study them) that have been developed for use in mice. This makes them the most appropriate system in which to study liver biology and cancer with relevance to clinical translation in human patients. Generally experiments will only be performed in adult animals to recapitulate liver disease in adult humans, however in some instances cancer initiation by be induced in juvenile mice as some frequently applied models require initiation at this time.

Typically, what will be done to an animal used in your project?

Typically, animals will have either a form of liver regeneration induced or cancers formed within the liver (latter typically by either via transplantation of cancer cells, or the induction of cancer driver genes through the injection of a viral vector system). We would then allow the cancer to grow and examine the effects of therapies (e.g. drugs given either via injection or ingested, or radiotherapy) upon the cancer during its progression. We also

examine potential therapeutic toxicity in conditions mirroring as close as possible to the human condition including treating tumours when there is background fat infiltration or scarring to the liver or use of other medicines which may promote liver damage (e.g. paracetamol). To measure these effects we will perform assessment of the tumour upon removal from the animal at a specific stage of tumour growth, but we may also measure tumour characteristics in the living animal
e.g. by imaging like CT scan as in human or by molecular imaging through microscopy; examining the tumour in situ in the mouse either under terminal anaesthesia or through the use of abdominal imaging windows. As liver tumours typically develop after chronic liver damage (e.g. fat infiltration) we may also model liver diseases (e.g. scarring or fat) through liver inflammation and/or aging. To study liver regeneration, areas of the liver may be damaged through chemicals or surgical removal and the regeneration of the remaining liver measured.

What are the expected impacts and/or adverse effects for the animals during your project?

Generally liver damage and/or cancer is painless in humans. The liver doesn't possess pain receptors in the same way the skin does for example. Liver capsular stretch causes discomfort in humans however. The liver is a key metabolic organ of the body and liver failure is manifested by a failure to produce or breakdown key nutrients or molecules (e.g. bilirubin causing jaundice). Weight loss is a typical sign of liver disease resulting from flu-like symptoms, loss of muscle bulk and reduced appetite and occurs over weeks to months and this occurs in both human and mice. Tumour models may in their late stage spread to other organs as metastases or even rupture within the liver.

In general the signs of the chronic mouse models including cancer models in mice are mild within our limits of detection. These may continue for months. Tumour formation usually takes 2 to 4 months. Mice are generally experience no symptoms during liver tumour formation. However, should rare consequences, for example tumour rupture occur, these effects are generally sudden and catastrophic with symptoms only experienced briefly. Where liver dysfunction or failure ensues the duration of symptoms is typically hours up to 2 days but it appears that this remains a painless process as it does 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)?

Most animals will have expected severity of no more than mild. Many mice will be sub-threshold, used for breeding or aging as controls. Most mice will undergo mild procedures only experiencing, for example, administration of a single injection and then treatments for cancer via ingestion prior to sampling before end-stage tumours form. A smaller proportion of mice will undergo moderate severity. These will include mice undergoing surgical procedures or repeated injections or the fitting of imaging windows or those extending to late stage cancer formation where symptoms may develop. Very rarely mice may experience severe side effects of tumours such as tumour rupture although we have become very experienced in successfully avoiding these sudden catastrophic events through careful animal husbandry and monitoring whilst developing these models.

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?

Therapies for liver disease and liver cancer need to take account of the multicellular nature of the organ. This includes the populations of cells which form scar (fibroblasts) and fight infections (immune system). Many of the current therapies for cancer treatment rely on the immune system to recognise and destroy a cancer. Treatments may also have off-target effects on other organs or cell types causing side effects to patients. Therefore, modelling the liver or cancer through cell culture in a dish can only take us so far towards therapy for patients as not all relevant cells are present in these systems. Using models which have intact immune systems which can respond to cancer and reflect the multicellular complexity of human disease are needed to generated effective treatments for patients.

Which non-animal alternatives did you consider for use in this project?

Where possible, we use cell culture models to study regeneration and cancer, rather than a living animals. Organoids and 3D models are excellent ways to study cancer cell growth and survival as well as stemness and invasion, and it is relatively easy to genetically manipulate the cells and apply drug treatments to inhibit/stimulate pathways in such assays. Much of our drug discovery work is done in these tissue culture systems for example.

Why were they not suitable?

In some instances cell culture models are suitable, however, for demonstration of efficacy of therapy or understanding complex biology a living organ and organism is required. Not all cells will grow under the cell culture conditions and it is difficult to completely model the influence of the tumour micro- environment (including interactions with immune cells) which requires interrogation in models with intact immune systems. Whilst improved invasion and metastatic assays are being developed, definitive proof in the ability of cancer cells to spread to other tissue sites, as well as spread into and out of the blood stream, is still best modelled in the whole organism. Drugs which are effective in cell culture may not be as effective in the whole organism if they can't reach the tumour, or are metabolically altered and so it is always important to take promising agents into the most appropriate in vivo model.
This allows the best possible reflection of the sorts of treatment efficacy and toxicity that humans may experience.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These 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 carefully design all our animal experiments. The numbers of animal used in experiments will be carefully predicted based upon data from previous published work by ourselves and other scientists. In all cases this will ensure that the minimum number of animals required for the experiment to give us useful data will be used but also reduces the likelihood that the animal experiment would have to be repeated in the future.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure that a minimal number of experimental animals are used experiments will be carefully designed by experienced researchers. We integrate pilot experiments with a small number of animals into cohort studies to minimise the likelihood of unexpected adverse effects related to genetic modification and/or therapy. By using the viral vector approach (akin to the AstraZeneca COVID-19 vaccine) we both minimise the biological variability of the model but also reducing breeding numbers required for complicated multiple genetic combinations which are reflective of the multiple mutations present in cancers.

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 regularly review our breeding colonies to ensure we are using the lowest number of animals to produce animals required for our studies. We will also perform transplant studies in wildtype mice, where relevant, thereby reducing our requirement for genetically altered animals. Where possible will share archived tissue and data from animals between experimental groups e.g. when controls from one colony can serve as controls for another experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the most up to date technologies that allow us to produce specific models in which the cancer develops in the correct tissue with as few as possible side effects. Where appropriate we will use mouse models using genetically altered mice to study liver regeneration and cancer. These models and methods have been refined to both mimic human disease whilst minimising harm, suffering, pain and distress to animals.

Why can’t you use animals that are less sentient?

Modelling adult disease requires, generally adult mammals as the most representative model system. It is widely recognised that genetic mouse models of liver disease cancer are most closely representative of human disease and tumours. The tumours follow a similar progression pathway as observed in human tumours; driven by related tumour drivers. This is done using mice that produce an enzyme to reduce/activate expression of the genes of interest only in specific tissues.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We routinely regularly monitor mice to ensure animal welfare is maintained and where necessary will institute enhancement monitoring of animals where welfare issues may occur. Where signs may or do occur appropriate action will be taken for example prophylactic analgesia will be given prior to procedures which may result in discomfort and additional analgesia may be used as required .

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow guidance from the ARRIVE guidelines and those from the Laboratory Animals Science Association.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We actively participate in updates both from the scientific literature, scientific meetings and specific training in animal models.

144. The roles of mutation and selection in tumour predisposition, growth and evolution

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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 Genetics, mutation-selection balance, cancer evolution

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the role of specific gene mutations in the development of tumours in transgenic mice models in order to understand more about the disease process, ultimately contributing to enhanced cancer prevention and treatment strategies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Genetic variation is critical to the development of cancer. That variation may be inherited or acquired. It is an ongoing task to explain whether genetic variants are drivers of tumour

growth or passengers. This distinction is critical for understanding and preventing or treating cancers. Carcinogenesis is a form of evolution in which advantageous (for the cancer) genetic changes cause the cancer cell clone to expend – in other word, they are selectively advantageous, usually in a Darwinian fashion. The proposed project will provide insights into fundamental principles of cancer evolution, specifically the roles of mutation and selection, and the problem of explaining why we observe non-random driver mutation spectra in tumours. Whilst mutational signatures and the underlying processes have been a recent area of excitement and progress in cancer genomics, mutational processes do not always explain the driver mutations we see cancers.

Additional to fundamental insights into tumorigenesis, this study has very important potential, indirect clinical benefits. Many of the tumours we will study, such as gliomas and AML, have a very poor prognosis. If we show our underpinning models of cancer evolution to be correct, we unlock the potential of new, powerful strategies. For example, instead of targeting the effects of a cancer driver mutation, methods to increase the effects of that mutation might paradoxically be more successful. For instance, hyperactivating a mutant oncogene could be toxic to cancer cells.

Mouse models allow the detailed study of those genes in all situations from their normal role in different tissues, the effects when the genes are activated or inactivated, and the possibility that the genetic variation produces vulnerabilities that can be exploited when preventing and treating cancer. Human cancer studies are essentially limited to observational studies tumours that present clinically and to the limited tests of new therapies in formal clinical trials. Mouse models allow exploration of multiple different scenarios, such as combinations of genes or the use of experimental treatments that will eventually find use in treating cancer patients or preventing cancer in the general population.

What outputs do you think you will see at the end of this project?

Our principal output will be publications reporting the effects of cancer-causing genetic variants in models, the underlying mechanisms, and the potential of targeting the gene and its related functions for cancer treatment and/or prevention in humans. We shall also seek to make our results available to a wider audience through the communication activities of the University.

Who or what will benefit from these outputs, and how?

The work will contribute to a broader fundamental understanding of carcinogenesis in the short term.

In the medium term, our work may suggest a restricted number of targets for therapy and/or prevention of cancer.

In the long term, our work might lead to benefits for human cancer patients or for anyone at risk of cancer.

How will you look to maximise the outputs of this work?

All new mice will be made available to others, initially to collaborators and then freely to all bona fide researchers.
We shall work with other groups with complementary expertise, e.g. high-throughput small

molecule screens to target our focus genes and their downstream targets.

All our work will be presented in publications or in lectures available to a wide scientific and general audience.

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.

Mice currently provide the only model of human disease that allows routine genetic manipulation, that has sufficient physiological similarity to humans, that comprises a large body of existing resources, and that can be bred with sufficient rapidity. Existing data on likely phenotypes and dosing of mice are far more comprehensive than those for other animals. Hence, precedents from the literature often allow us to anticipate phenotypes. For example, we have considerable experience in identifying the signs and symptoms of intestinal tumour burden, including both early signs that do not cause distress or automatically activate killing (e.g. pallor) and signs that require killing (eg hunching, weight loss, lethargy). Endpoints in mouse cancer models are generally well established in the literature and the applicant laboratory. Several mutant mice with well-established phenotypes are already in the laboratory. Use of imaging in mice, where this is practicable, will also allow us to predict more accurately when an animal may begin to experience distress so that it can be killed before external signs become apparent.

Existing mutant mice will be procured from collaborators or commercial sources, thus reducing the breeding needed to set up a new mutant stock. New mutant models will be generated by commercial providers or by the local transgenics facility and transferred to this licence only once established.

Treatments or prophylaxis can usually employ existing data on administration routes and doses which mean that mice can be treated relatively rapidly and with minimal side-effects. If these are not available, we shall scale dosages and determine routes of administration using pilot toxicity experiments in small numbers of mice (typically 2-3 per regimen).

Whenever the administration of gene inducing agents is required in utero or young pups we will carry out a series of pilot experiments. We will begin with the least invasive method and latest timepoint and work backwards, only using prenatal administration and/or more invasive methods if absolutely necessary. CRE/FLP recombination frequency, phenotypes and adverse effects will be assessed and completed before proceeding to earlier timepoints or larger scale experiments.

Typically, what will be done to an animal used in your project?

The typical experimental mouse will be bred to carry one or more genetic changes of interest in a conditional, inducible state. These will be activated by tamoxifen injection as a young adult. The mouse will be aged until a set time point or, if the phenotype has not

been established until it develops symptoms of tumours for up to 18 months. It will then be killed and its tissues used for assessment of the tissue, cell and molecular phenotype.

What are the expected impacts and/or adverse effects for the animals during your project?

As some of the mice are models of human tumours, they may develop symptoms similar to those associated with human patients with the equivalent disease. Some of these may be non-specific (e.g. weight loss, anaemia), others may be caused by particular cancer types (e.g. brain tumours may cause changes in behaviour), and others may be directly indicative of tumour development (e.g. the appearance of lumps within organs or under the skin, liver enlargement).

Expected severity categories and the 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 (75%)

Moderate severity (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?

Whilst mice do not provide a perfect model of cancer, they represent an acceptable compromise between resemblance to humans and difficulties in maintaining colonies of larger mammals. In addition, the ability to transplant mouse and human tumours into selected mice provides an opportunity to examine the effects of anti-cancer therapies or prophylaxis.

Which non-animal alternatives did you consider for use in this project?

Cancer cell lines, human organoids, mouse organoids

We use all of these models alongside mouse models and utilise them wherever feasible. For example, when testing the potential effects of new anti-cancer treatments, cell and organoid models provide the means to exclude many compounds without activity rapidly, thus allowing a focussed range of potential therapies to be used in animal models.

Why were they not suitable?

Tumour growth often relies critically on the interaction of multiple cellular and/or tissue compartments. In fact many cancer predisposition genes do not act in the cancer itself, but

in the tissue microenvironment (e.g. immune cells, fibroblasts) or even distantly (e.g. liver detoxification of carcinogens). Animal models are therefore required to faithfully model many cancers. In addition, non-mammalian animal models generally fail to recapitulate critical features of human tumorigenesis, including the hormonal and immune 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 estimate is based on the numbers required for a similar level of experimental activity over the past 10 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All animals are genetically tested straight after weaning and surplus animals killed by a schedule 1 method or used as controls in other experiments. For simple binary traits of new models, we shall aim for the minimum required (10 mutants and matched littermates without the mutation). For molecular phenotypes, such as gene expression analysis, a typical experiment would aim to detect a 1.5-fold change in gene expression at P=0.0001 (reflecting multiple tests), requiring 45 mice in each of test and control groups to provide 80% power. For clinical interventions, such as testing anti-cancer agents, we power to detect a larger effect size (e.g. 2-fold reduction in tumour burden) with strong statistical evidence (P=0.001) and high power (>95%), requiring 20 animals in each arm. In some studies, mouse and in vitro data can be mutually supportive (e.g. assessing tumorigenicity in mouse xenografts and human cell lines), thus reducing mouse numbers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding plans are designed to be efficient using the CAMplanner tool.

All new mutants are aged and examined at different ages in pilot experiments so as to set endpoints for full assessment.

Mice are bred onto identical genetic backgrounds to minimise unimportant genetic variation in the trait of interest.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 models used with carry different genetic changes implicated in human tumour growth. The procedures required will be minimal (essentially limited to induction of mutations using additions to water or food, or using tamoxifen injections or similar). Most of the models we study will only have mutations induced as adults and will therefore develop normally.

Why can't you use animals that are less sentient?

Whilst there are some exceptions, models of human cancer have consistently proven to be most successful when mammals are utilised. Mice are the most appropriate such animal owing to rapid generation time, established resources such as methods of gene editing and activation, and reasonable similarity to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be monitored closely in pilot experiments that will indicate the time period for enhanced monitoring in full experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

https://www.nc3rs.org.uk/experimental-design https://acmedsci.ac.uk/policy/uk-policy/animals-in-research

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?
Through the 3Rs website: https://www.nc3rs.org.uk and Newsletter.

Laboratory staff will be advised of any relevant changes and instructed to implement them. Any changes to protocols as a result will be implemented via licence amendments.

145. Musculoskeletal Tissue Regeneration and Repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Musculoskeletal, Regeneration, Stem Cells, Bone, Cartilage

Animal types	Life stages
Mice	adult, aged, juvenile, embryo, neonate, pregnant
Rats	adult, aged, juvenile
Sheep	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve the regeneration and repair of musculoskeletal 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?

Diseases of the bone and joints account for 25% of GP attendances and consume a major proportion of hospital resources. Each year in the UK alone, over 1 million fractures occur and over 100,000 joint replacements are performed, many of these caused by osteoporosis and osteoarthritis, respectively.

This major burden of disease will rise further as the age of the population increases. There is therefore a burning need to increase our understanding of these disabling conditions and to investigate regeneration and repair of bones, joints, muscles, ligaments and tendons. One of the major functions of the body's system of bones and joints is to exert forces and respond to loads. However, how the body interacts with the physical world is poorly understood. Yet this has a major role in the cause of diseases of the bones and joints such as arthritis and osteoporosis. In order to reduce pain and disability, there is also a need to look at improving healing after fractures, especially in young patients who have suffered major trauma and older patients who have suffered osteoporotic fractures, or fractures due to systemic cancers.

What outputs do you think you will see at the end of this project?

The primary output will be generation of knowledge that will be largely disseminated by publication in peer reviewed journals and presentation at conferences, seminars and workshops. Components of the work may also be suitable for public engagement events to increase the public's awareness of our work and its implications for health. In addition, I expect the work from this licence to form the justification for clinical trial of stem cell therapy in certain musculoskeletal conditions.

Who or what will benefit from these outputs, and how?

The work in the project licence is designed to help people with musculoskeletal disorders, especially conditions that damage patients joints and situations where there is impaired bone repair after fracture.

How will you look to maximise the outputs of this work?

The work will be published in peer reviewed journals and presented at peer reviewed meetings. To ensure maximum dissemination, journals with gold open access will be prioritised. To ensure dissemination of all new knowledge and prevent unknowing and unnecessary repetition of experiments by others, I will seek to publish all data generated under this project including negative results, again utilizing preprint repositories such as bioRxiv.

The work on novel cutting technologies is part of a consortium of 5 universities and all of the work (successful and unsuccessful) is shared with them. In addition, as part of this research council work we have canvassed viewpoints from different specialties in the colleges of surgeons and will run workshops on the findings.

Species and numbers of animals expected to be used

- Mice: 200
- Rats: 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.

All the procedures have been carefully refined in order to minimise discomfort. Most of the techniques described in are similar to those used in patients, in particular, the cutting of bone, stabilising the cut bone with an external frame around the bone and gradually moving apart the 2 pieces of bone. Placing a pin within the bone to stabilise a fracture is also very common. We know from the feedback from patients that these procedures are well tolerated. The majority of the work will be done in adult rats and mice, in which we will cut or fracture a bone under full anaesthesia and study the repair mechanism for the bone. The animals will have the same care as medical or veterinary patients with bone problems.

We will also study situations where the cartilage is damaged e.g. arthritis and ways to repair this. The choice of species and strain to be used depends on the study. Some of the procedures were originally developed in rabbits. The bones of this species are quite large and allow precise control of the mechanical environment. However, in line with the principle of minimising the use of large animals, we have validated these models in the rat and in some cases it has been possible to go down to the mouse; particularly if using a pin within the bone. However, stabilising the bone with devices around the outside of the limb are extremely challenging in bones of these dimensions.

Typically, what will be done to an animal used in your project?

All the procedures have been carefully refined in order to minimise discomfort. Most of the techniques described herein are similar to those used in patients, in particular, the cutting of the bone, fixing the 2 pieces of bone with an external frame or internal pin, and then gradually moving these pieces of bone apart. We know from the feedback from patients that these procedures are well tolerated.

The operations typically last between 20 minutes and 1 hour. Most animals will undergo a single procedure but some animals will have a second procedure for instance to inject stem cells a few weeks after their initial bone cutting procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

Deaths resulting from anaesthesia or surgical complications are uncommon (<1%) and will be minimised by correct dosing of anaesthetics, by accurate weighing and by maintenance of body temperature during procedures.

Pain will be controlled during surgery by general anaesthesia and post surgery by analgesics as advised by the veterinary service. Judging by the early return of mobility, the pain has settled within 2 days of the procedure.

Post-surgical infections can occur in about 1% of animals. Risk of infection will be minimised by good surgical and aseptic techniques. Surgical sites will be monitored for signs of inflammation and infection. Antibiotic cover will be given under the advice of the veterinary service if required.

The skin wound may breakdown (estimated rate <5%). If it does, it may be repaired on one occasion only in consultation with the veterinary service.

In the studies examining whether stem cells can help fight infection, organisms will be introduced into the bone defect, but to mimic the clinical situation, only localised infection

will be created.

In the studies examining cartilage damage (due to various causes including infection) and its repair, a localised 'arthritis' in a single joint will be created, which will not significantly affect mobility.

Best practice guidelines for surgery/post-surgical care, anaesthesia and analgesia will be followed at all times.

Mobility is not expected to be affected by these procedures, however, the animals will be closely monitored for any signs of inflammation or adverse reactions in the surgical areas. The majority of animals mobilise freely within 2 days. If there are any concerns, veterinary advice will be sought and taken.

Significant Weight loss is uncommon (<2%) but will be closely monitored.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of the animals will undergo procedures that mimic those happening to patients. In summary (1) musculoskeletal tissues will be cut using novel devices such as ultrasound, (2) studies will be carried out in which bone will be cut under anaesthetic and stabilised with internal or external devices and (3) lesions will be created in cartilage and bone and the repair of these defects will be studied to improve the treatment of arthritis and bone loss and bone metastasis. These are of moderate severity. Approximately 5% of animals may be used for establishment and maintenance genetically altered animal breeding programmes or for production of genetically altered mice that will simply be used for harvesting tissues. These animals will fall into a sub-threshold limit of severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Some of the research questions can be answered in patients or in cell culture models or in engineering models, however in some situations, such as fracture repair, animals have to be used because the response of the whole animal has to be studied. Frequently this cannot be studied in patients as (a) there is too much patient variability, particularly with respect to the trauma/ fracture configuration and (b) the bone strength has to be measured which involves fracturing the bone post mortem. In addition in many experiments the tissues being investigated have to harvested and sectioned for immunohistochemistry.

Which non-animal alternatives did you consider for use in this project?

The work outlined in this project license has all been carefully planned in line with the principles of replacement, reduction and refinement. Wherever possible, animals will not be used.

- Cell Culture: For instance, if the answer to the question can be obtained from cell culture, these techniques will be employed.
- Ex Vivo Models: In parallel with this, we have appropriate ethical approval in place to permit us to use (with patient consent), human surgical discard material. In particular, we have used discarded femoral heads to develop an ex vivo model for cartilage repair.
- Finite Element Engineering Computer Models: Further, in some situations, we are getting suitable data direct from patients either from measurements from CT scans. This has been coupled with mechanical data obtained in the laboratory and fed into complex ("finite element") computer models.

Why were they not suitable?

There is a need to see the healing response in the animal; to examine this sawbones, cadaver, FE models, explants and cell culture studies are not adequate.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 experiments where all of the baseline parameters are known power calculations will be done however in some cases some preliminary work needs to be performed to establish the baseline parameters (e.g. Inoculum of bacteria). Our previous work has indicated the approximate numbers of animals that are required per experiment to give sufficient power. Assuming a type 1 error or 0.05 and a type 2 error of 0.1 with the standard deviations from our previous work it would suggest sample sizes of the order of 4 to 10 would be needed to observe a difference of 20% for the bone, muscle and cartilage regeneration and repair experiments. The novel cutting technology experiments are of a proof of concept nature and therefore only limited numbers will be required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The numbers of animals used will be kept to the minimum necessary to enable statistically sound conclusions to be drawn from the studies. The numbers requested reflect this and are, therefore, the minimum number required to achieve the important objectives.

To ensure best practice in statistical analysis and experimental design all new staff members working under this license will attend the in-house 'Experimental Design Course'. Planned experiments are discussed regularly within group meetings to ensure all

are correctly controlled and to facilitate sharing of tissues/data for the most effective use 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?

The conditions will be standardised, the outcome measures will be refined and where appropriate inbred strains will be used. Our experiments are also designed to reduce the number of variables to as few as possible and thereby reduce the number of control groups required. In this respect, we will always consider carefully whether it is important to include ‘naïve’ as well as ‘vehicle’ control groups in experiments, or if the latter alone is sufficient for interpretation 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.

I am committed to ensuring the most refined protocols are used in our studies. To this end, all the procedures have been carefully refined in order to minimise discomfort. Most of the techniques described in this project licence are similar to those used in patients, in particular, the cutting of tissues and stabilisation of the bone using devices that are placed inside the body or ones on the outside of the limb (Protocols 1,4,5,6,7). We know from the feedback from patients that these procedures are well tolerated.

Arthritis, including the type due to bacteria, occurs spontaneously in human and veterinary patients and causes discomfort if untreated. The procedures looking at improving the treatment of these conditions (protocols 2 and 3) have been designed to minimise pain and distress. Furthermore, these animals will be monitored extremely carefully to ensure that the animals are always in a satisfactory condition.

Protocol 7 is a refinement of historic bone tumour models, whereby animals were given systemic cancer, resulting in widespread metastasis and the symptoms thereof, with some of these seeding to the femur. Our technique of direct intra-osseous injection has been described by many research groups and results in a solitary tumour within bone, that does not metastasise. Therefore, this reduces harm to the animal, whilst maintaining useful biochemical and morphological parallels with human disease.

Why can’t you use animals that are less sentient?

The work will be done in rats and mice, in which we will divide a bone under full anaesthesia and study the repair mechanism for the bone. The animals will have the same care as medical or veterinary patients with bone problems. We will also study situations where the cartilage is damaged e.g. arthritis and ways to repair this. The choice of species and strain to be used depends on the study. Some of the procedures were originally

developed in rabbits. The bones of this species are quite large and allow precise control of the mechanical environment. However, in line with the principle of refinement, we have validated these models in the rat and in some cases it has been possible to go down to the mouse. However, external fixation is extremely challenging in bones of these dimensions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In line with local policy, we will adopt the latest techniques in animal handling (eg cupping) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied.

The novel cutting technologies (e.g. ultrasound, laser) that will be evaluated are being investigated to try and refine surgical procedures.

Anaesthesia and analgesia will be provided where suitable (eg for humane restraint, during and recovery from surgery). Where ever possible local anaesthetic will be used at the operation site. Post operatively the animals will be monitored very carefully and given post-operative analgesia.

To reduce infection risk, the best aseptic technique will be used during surgery (eg sterilization of instruments between animals, full surgical drapes) and immunocompromised mice will be housed in IVC cages.
Infection experiments will carefully titrate the number of organisms to identify the lowest number of bacteria required for the research question, or use early endpoints that prevent animals experiencing more severe harms.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
Our institute employs a dedicated team of veterinarians that are continually seeking to improve animal welfare and refine animal use. My group consults closely with this team and takes full advantage of the extensive resources provided on their website to ensure we are following current best practices. These resources include comprehensive guidelines and standard operating procedures for most common rodent procedures. We have also consulted the NC3Rs research strategy paper by Prescott MJ, Lidster K (2017).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute employs a team of dedicated veterinarians that are continually seeking to improve animal welfare and refine animal use. Both I and my group consult closely with this team and take full advantage of the extensive resources provided on their website to ensure we are following current best practices. These resources include comprehensive guidelines and standard operating procedures for most common rodent procedures that are continually being updated. Our university is also in the process of adopting the improved rodent handling methods that reduce animal stress (detailed by Hurst et al. Nat Methods 2010) and our animal facilities now provide environment enrichment as standard. My group will adopt these methods alongside the staff in our animal facilities. We will also take full advantage of the annual 3R’s seminar day organized by the University’s Animal Welfare Committee to find out about pioneering developments in best practice.

146. Acoustic tagging and tracking

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

Salmon, Acoustic, Tagging, Lamprey, Weir removal

Animal types	Life stages
Atlantic salmon (Salmon salar)	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 fish behaviour during both downstream and upstream migrations around artificial structures to investigate if the operation of the structure is having a negative impact on the species population. Additionally, where remediation work is carried out at structures, to demonstrate the positive impacts of the 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?

The species that are listed under the two Special Areas of Conservation (SAC) that this project focuses on are all in an unfavourable conservation status. This is based on their current population numbers and range following assessments carried out every 6 years. The primary objective of the management of these species is to achieve Favourable Conservation Status (FCS). It is vital that we understand the impacts of man-made features in the river on these species, so we can mitigate for it with the intention of returning the species to FCS. It is also key to understand the dependencies of predators (pike) and how they impact on protected species in an artificial environment.

The operator is an evidence led body, and we must base decisions based on best available evidence. Where there is an evidence gap, this should be identified, and then addressed if possible. This project aims to fill an number of evidence gaps and provide robust evidence for future changes to operations , as well as understanding the impact of structures on a wide range of species. It is key to also share this information with others to ensure best practice is disseminated.

The types of structures we would be interested in include weirs, sluice gates and artificial structures within the river. These can all have an impact on passage success as they create unnatural flows, rapid velocity changes, and higher velocities than natural, making passage difficult for fish species.

Unnatural flows could include waterbodies being backed up through sluices gate operation (effectively holding water back and forcing it to flow 'upstream' due to flow restrictions at the sluice gates). This would make downstream migration potentially challenging as fish travel the wrong way.

Rapid velocity changes are associated with sharp crested weirs and sluices, and create conditions that fish are reluctant to pass (preferring gradually increasing velocity changes as would be experienced naturally, rather than sudden rapid changes). This would make downstream migration challenging as fish are held up above structures, and the issues associated with delay (energy consumption, increased risk of predation and potential to miss 'window of opportunity' for sea entry).

What outputs do you think you will see at the end of this project?

The outputs include:

Improvement in scientific understanding about fish behaviour around structures.

Better operation the structure for the benefit of all target species to maximise passage and therefore survival. Understanding of the predator/prey interaction as a result of the structures provides robust evidence for negative impact on the SAC species.

Better understanding of fish behaviour to ensure future designs and operation of structures causes the minimal amount of disruption to migratory fish species, within the UK and wider as applicable.

Who or what will benefit from these outputs, and how?

These outputs will benefit:

The local populations of SAC (salmon, shad, river lamprey, sea lamprey) and non SAC species (grayling) in the immediate and long term.

The wider range of each of the SAC species through dissemination of results in the medium to long term.

Relevant organisations that are dealing with similar situation on structures worldwide in the medium to long term

Changes to the operation of the structures through either water release patterns or

operational changes, or where possible full or partial removal of the structures.

How will you look to maximise the outputs of this work?

We are disseminating results from previous work through local fisheries groups and using it to influence future operations. Additionally through projects we are collaborating with organisations such as the River Restoration Centre, Dam Removal Europe and Universities to disseminate the results. This will be through publication of papers and reports.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used are those that are present in the catchments identified and are of concern through their designation as species that are highlighted under each SAC. Each SAC has a number of species and/or habitats that it is designated for, either through a primary reason for designation); or qualifying features. ,). We are proposing a range of species and lifestages to understand the implications for different species. Different species have different swimming capabilities, and these capabilities change during the fishes life cycle. All of the fish focused under this project licence are anadromous, in that they hatch in freshwater, before migrating to partial or full salt water for a period of growth and maturation, before returning to freshwater to spawn again and complete the cycle. Most salmon, river lamprey and brook lamprey spawn a single time, whereas thwaite shad make this journey multiple times through their lifespan and spawn multiple times.

On these journeys, any barriers can provide issues that fish may find difficult to pass. This difficulty in passage may manifest itself in delays, milling behaviour, extended periods of failed attempts and energy reserve losses associated with repeated attempts.

We are focusing on the following species and life stages:
- Adult salmon migrating upstream.
- Adult river lamprey migrating upstream
- Adult sea lamprey migrating upstream
- Juvenile salmon moving downstream

In addition, pike are included as they predate on salmon smolts. Physical structures within the river can create habitats that favour predators over riverine species, so it is important to understand if this can be mitigated against if it is proven to be an issue.

Typically, what will be done to an animal used in your project?

Each fish will be caught by a specific method, anaesthetised and then the fish will be tagged with an acoustic tag. From previous experience, tagging typically takes less than 2

minutes. We would expect to be tagging the following maximum number of fish:

200 Atlantic salmon (Salmo salar) smolts per year (capture method - modified fyke netting)
40 grayling (Thymallus thymallus) per year (capture method - rod and line)
50 sea lamprey (Petromyzon marinus) per year (capture method - permanent fish trap) 50 river lamprey (Lampetra fluviatilis) per year (capture method - permanent fish trap) 50 adult Atlantic salmon (Salmo salar) per year (capture method - permanent fish trap) 20 adult Pike (Esox lucius) per year (capture method - rod and line)
30-60 adult Thwaite shad (Alosa fallax) per year (capture method - rod and line or modified net)

Post surgery, all fish will be retained in a holding tank in the river until they are behaving normally as assessed by experienced fisheries staff. They will then be released into the river at the site of capture, with untagged individuals.

What are the expected impacts and/or adverse effects for the animals during your project?

Tag size will be minimised to ensure the tag burden for each fish is within recommended levels. Following tagging, fish will be retained for a short period to ensure they are behaving normally prior to release.

Tagging will be carried out be experienced staff. Over the past 5 year project licence, no fish have died as a result of the regulated procedure. Recaptures of a small number of fish have shown no issues with the wound or behaviour.

There would be some pain for the fish following tagging, but this will be minimised through careful handling and surgery carried out by experienced staff.

At the end of the regulated procedure the fish will be released back into the wild to complete its lifecycle with no expectation of long term negative effects upon the fish.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?
Expected level of severity is moderate for all species.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project must use the animals described to ensure natural behaviour of fish at the different structures/barriers within the rivers can be assessed.

Which non-animal alternatives did you consider for use in this project?

Particle tracking models can be used to estimate behaviour where currents and ‘normal’ fish behaviour are fed into a program to estimate fish behaviour.

Why were they not suitable?

These models however do not take into account the full range of stimuli that are present and all the other factors that may influence survival (for example increased risk of predation associated with a delay), or the actual behavioural characteristics displayed by fish when complex flows are exhibited, such as if fish did enter a lake where they may or may not find the exit point to go down river due to anthropogentic impacts on the natural flows within the lake.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 figures are based on expected projects and numbers per project as well as prior experience in tagging. We may have additional questions raised during the project that require more fish to be tagged, or other locations in future.

Prior experience and knowledge from other organisations will also provide knowledge of how many fish to tag.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In 2015 we tagged 94 salmon smolts under a previous licence. During this project the majority of the fish were tagged on a small number of night (54 on one night). Having reviewed the results from 2015, it would be more beneficial if fewer fish were tagged each night, but over a wider range of flow conditions. Therefore, for smolts, we will be tagging in the region of 60 - 100 fish in each of the study years, but planning on tagging over more nights.

Sea trout may also be affected in the same way as salmon smolts by the sluice structure, however, by focusing on salmon smolts, we can use these results as a proxy for sea trout to afford them the same protection, both at this structure, and in locations where salmon are not present, but trout are.

By using larger tags in larger fish, we will have greater battery expectancy and therefore potentially be able to reduce how many fish are tagged as they will survive and the tags be operating over more than 1 year.

For river and sea lamprey 2021 will be a trial year for the project and future assessments

will be made based on results of tagging in 2021. For sea lamprey, they migrate in a smaller window than river lamprey, so we may need to tag fewer individuals, as it is not critical to cover a wide range of environmental conditions (river flow for example). It would be expected that a maximum of 50 fish per year would be tagged as they all migrate at a similar time of year so there is limited variability in flows that need to be taken into account, therefore reducing the number of fish needed to be tagged.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Battery size and therefore life expectancy, is one of the biggest limiting factors for acoustic telemetry. We will ensure that we use the most suitable tag for battery life expectancy to ensure the maximum amount of data is collected, whilst not impacting on fish survival and remaining within recognised weight limits of tags v's body weight.

The use of programmable tags that 'hibernate' for a predetermined amount of time, before turning back on will allow us to cover at least 2 spawning cycles for shad, depending on trials and survival rates, we may well be able to reduce the number of fish tagged in a specific project. For example. once we have tagged 20 pike in the target waterbody, if survival is high, we would have fish in the system for a longer than expected period of time and therefore be able to reduce the numbers tagged in subsequent 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.

Acoustic tagging of fish is the model used throughout this study.

Once an acoustic tag has been inserted into the fish, it transmits a unique identification code on a pre- determined time frequency that is detected when within range of an acoustic receiver that is placed in a fixed location within the river. The data gathered from the receivers is either done in real-time or at a later date once the receivers are recovered, depending on the receiver type. Once the data is analysed, it is possible to determine travel speeds, delays and fine scale movements within the river system.

Why can’t you use animals that are less sentient?

Monitoring wild fish is the only way to provide the most robust evidence that is required to challenge the current operations for the benefit of the SAC species previously mentioned. It is vital that live animals, that will behave in a natural way be used in the experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Use of specialist equipment to reduce stress on fish when being trapped

Tagging on site at the designated POLE – no transportation results in a reduction in stress and handling the tagging methods used involves the least pain, suffering or lasting distress to the individual fish tagged. Within their life history, fish cope very well with a vast amounts of physical damage (predator attacks and migration/spawning damage for example).

Tags will be the VEMCO V5 acoustic tag, which will not exceed the 4% rule (Winter 1983) used when tagging fish (the weight of the tag in air will not exceed 4% of the fishes body weight in air).

It is vital to the project that fish behave as naturally as possible post tagging/release. Therefore the following will be followed:

- Tagging methods that have been shown to have little or no impact on fish behaviour will be followed (e.g. as described by Garner et el 2015).
- Tag will be used that are suitably sized for the fish being used as researched by the tag provider (VEMCO)

Fish will be monitored in recovery tank to ensure that their behaviour is normal before being released back to the river, with other fish that have not been operated on to afford protection due to the natural shoaling behaviour of smolts.

Where required we would monitor water temperature to limit the impact on cold water species (for example, not catching grayling by rod and line if the water temperature is above 15c).

Work undertaken in low light conditions to least disturb the fish, prior to anesthetising the fish.

Latest refinements in tag battery life and size will be used to ensure the most appropriate tags are used to ensure good data collection. Sea lamprey tagging methodology has been researched through discussions in Canada with fisheries staff tagging fish there. The most appropriate tags will be used to get the largest data set possible from each tagged fish.

Guidance from other studies where grayling have been tagged with acoustic tags will be reviewed and discussions with researchers will provide guidance on latest refinement techniques.

Vidalife (or similar) to be used on all surfaces fish will come into contact with to limit mucus loss. During angling as a capture method, we will ensure that:

- Grayling and pike are not caught by this method when water temperatures are above 15c
- All fish are given long recovery times in the river either in landing net or keepnet (until opercular movement is normal) as assessed by an experienced member of staff)

We are committed to using analgesia to limit post-surgical pain in all fish that are tagged surgically which will be discussed with our Vet.

What published best practice guidance will you follow to ensure experiments are

conducted in the most refined way?

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How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will liaise regularly with our vet and implement any emerging best practise. For example, during the last project licence, we started irrigating the gills of the salmon smolts during surgery, which provides a clear benefit in terms of fish welfare.

Staff carrying out the work are all highly experienced and will all ensure that where new practises are emerging, we will implement them.

147. Cellular degradation mechanisms in the hematopoietic 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

autophagy, immune system, ageing, metabolism, blood cancer

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

With this project licence we aim to fill a major gap in our understanding of the mechanisms by which autophagy has an impact on the health of blood cells, and identify drugs to decrease or increase levels of autophagy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 found that autophagy, the cell's major way to degrade and recycle, is essential for the fate and function of healthy blood cells. Autophagy helps blood cells to mature, it prevents their ageing and death, and thereby plays a key role in disease. While our previous work

demonstrates key roles for autophagy in blood cancers, ageing of the immune system, and resolution of inflammation (such as in inflammatory bowel disease), we do not know what autophagy degrades and provides to the cell.
Understanding the molecular basis of autophagy's role in disease will help us to design drugs to improve stem cells (e.g. regenerative medicine), improve the immune system of older adults (e.g. immune response to infectious diseases and vaccination responses), and help us to design drugs for chronic age-related diseases (e.g. osteoarthritis and osteoporosis).

What outputs do you think you will see at the end of this project?

In the next 5 years the challenge in this field lies in understanding the mechanisms by which autophagy, the way by which our cells degrade and recycle is involved in health and disease of the immune system. Autophagy levels decline with age and this work addresses the molecular and cellular consequences of this phenomenon. Low autophagy and the ensuing increased production of inflammatory soluble factors contribute to some of the main ageing diseases, e.g. neurodegeneration and cardiovascular diseases. However, we do not understand yet which cells mostly contribute to this ‘inflamm-aging’ and how. This is the purpose of these experiments. The knowledge of the molecular physiology of ageing will in the short term inform other scientists in the ageing field and in the long term inform the design of therapies to combat diseases prevalent in the elderly.

1st output: publish our findings

We have elucidated the relationship between autophagy and an inflammatory disease with its underlying mechanisms. Apart from publishing our work, we are hoping to identify new modulators of autophagy to dampen inflammation. We have elucidated the signalling for autophagy in hematopoietic stem cells involved in maintaining them, with a manuscript in revision.

The knowledge gained in the immune system will be of interest to a wide range of scientists, as we expect our findings to be broadly if not universally applicable to other tissues. With all our work we are aiming for publications with high impact.

2nd output: find novel drugs

Based on the knowledge we obtain from this licence, we hope to generate novel drugs and re-purpose existing drugs to improve vaccinations, immune responses in the ageing population and age-related diseases. From the COVID-19 pandemic we know how important this is. We will take our discoveries made in mice into human clinical trials. The aim is to deliver the autophagy-modulating drugs to man.
We have conducted a clinical trial to test if autophagy induction improves immune memory resposnes and are currently asking for ethical approval to conduct a small experimental human trial on influenza vaccination in older adults.

3rd output: understand role of autophagy in the pathogenesis of disease
Osteoarthritis is very common in the older population without a treatment that may halt or reverse the disease. With this work, we will make progress towards identifying new molecular pathways that can be targeted with drugs. The IP of new targets will be patented. We expect to be testing newly identified drugs in a small experimental medicine trial in humans within the timeframe of this licence.

Multiple myeloma (MM) is a cancer of a blood cell called B lymphocytes. Over the course of the disease, blood cells are made in excess in the bone marrow, while the bone is being destroyed. The drug we are working on is FDA approved for another disease and inhibits the formation of bone- resorbing cells, thereby conserving the bone in multiple myeloma. We are aiming to understand its precise mode of action (we found it is via autophagy) and whether it has a similar effect on human bone. With this knowledge we are aiming in the next year to apply for a grant with a commercial partner to develop this drug or a drug with similar chemical properties for MM and osteoporosis.

4th output: generate new mouse models to be used by others

With this licence we are developing new mouse models, for example 1) Transgenic mouse model, which can be used to determine the autophagic cargo in any type of cell in the body, for example cancer cells, 2) reporter mice will help us to understand if molecules in the autophagy pathway can be used as a biomarker for ageing. Early biomarkers are essential for the development of drugs aiming at halting aged-related disease in humans, as waiting for ageing hallmarks or disease to measure efficacy will take too long. Once published, we will make mouse models available to the scientific community via repositories or sending sperm/embryos.

Who or what will benefit from these outputs, and how?

Communication with the scientific community The findings from this study will be presented at national and international scientific meetings (the applicant is invited speaker at about 10 international conferences/ year) and in high profile publications (1 per year). Findings will be made available to other scientists through publication in peer-reviewed journals, on preprint servers, at scientific conferences and meetings. Under the previous licence my team published 6 major papers and contributed to another 20.

The beneficiaries are (A) the elderly by improvement of their immunity (within 5 years), and (B) UK society and economy as a whole by improving vaccine efficacy in the elderly (within 7 years), as vaccine efficacy in older adults is notoriously low, and poor responses to coronavirus in the elderly has caused major disruption to the world in 2020; (C) patients suffering from osteoarthritis or multiple myeloma by extending their treatment options (within 10 years).

How will you look to maximise the outputs of this work?

We will collaborate with other researchers who have requisite expertise and knowledge to maximise outputs. We will speed up dissemination of knowledge through preprint servers and deposit our work on symplectic before publication. We will only publish in open access journals. We will include unsuccessful approaches together with the successful approaches. We will share our knowledge with our collaborators as soon as possible and before publication. We will present unpublished data in scientific conferences.

Species and numbers of animals expected to be used

- Mice: 23000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Firstly, ageing research cannot easily be done in vitro : (1) other lower model organisms such as flies or worms do not show the same ageing hallmarks and cannot mimic age-related diseases. Mice are considered the most suitable model of mammalian ageing, having been extensively utilised as such by the scientific community. A large amount of literature, data and models are available for use and comparison. Furthermore, the use of mice is feasible to study mammalian ageing due to their relatively short life-span.

Secondly mice are also the most suitable species for the study of the immune system (inflammatory diseases and response to pathogens) as genes can be easily knocked out or inserted allowing the possibility to interrogate a pathway leading to disease.

Typically, what will be done to an animal used in your project?

Genetically altered to modify autophagy, given agent (e.g. virus, pro-inflammatory substance, tumour cells) to induce inflammation usually by a single administration (typical in the drinking water or by intravenous injection), then blood sampling twice over the course of the disease, then killing humanely.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice used in experiments, would typically experience mild transient discomfort when infectious organisms or test substances are injected and when blood samples are taken. Animals are also expected to develop illness as a result of infection including diarrhoea, starry coat, huddling, weight loss and reluctance to move usually for no longer than 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)?

This animal licence only includes mice as a species.

It contains 3 breeding protocols and we expect the following severity (protocol 1) 100% of mild severity or less (protocol 2) 50% of mice with moderate severity (due to the breeding strategy, 50% of mice on average will have normal genotype) and (protocol 3) 20% of moderate severity as the majority of animals will not show any adverse effects or will not be treated.

In addition it contains 7 experimental protocols, all with moderate severity. In protocols 4, 9 and 10 we expect 50% of mice to experience moderate severity and the remaining 50% will experience mild (or less) severity of symptoms , e.g. because they are controls, littermates, or have not undergone procedures with long lasting mild pain or short term moderate pain, such as irradiation. In protocols 5-8 we expect 100% of animals to experience a 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?

Firstly, ageing research cannot easily be done in vitro : (1) the process of ageing cannot be mimicked in a dish

Secondly, immune responses cannot be recapitulated in vitro, as it involves interaction between different cell types and requires specific anatomical locations. Rodents are the lowest vertebrate on which well-established models of immune responses of interest have been developed.

Thirdly, there is no in vitro technique to replace the entire differentiation of blood cells from a stem cell.

Lastly dose, kinetics, side effects and tissue distribution of a novel drug need to be tested in an animal before its development as a drug for humans.

Which non-animal alternatives did you consider for use in this project?

We are complementing or replacing animal experiments with human cell culture models whenever possible. Examples of this in our research are the use of blood samples from ageing donors or from vaccinated donors, fat tissue from atherosclerosis or inflammatory bowel disease patients and using human samples to do the drug and genetic screens. However, it is very difficult to do mechanistic studies in primary cells obtained from humans, as we cannot genetically delete a pathway (such as autophagy) or examine the development of a disease as samples are obtained from patients with ongoing disease. It is also notoriously difficult to obtain healthy samples (for example biopsies) from humans for comparison.

In parallel to mouse models, we set up cell lines with the same genetic modification to address molecular mechanisms. These are alternatives to animal experiments. While these in vitro experiments are time-consuming to set up and do not always mimic what is happening in vivo, when they do we work with the replacement model. However, we still need animal models as most of our experiments investigate autophagy or cellular metabolism which cannot be mimicked in vitro, where cells bathe in high levels of glucose, amino acids and lipids to keep them alive, which by itself alters the status of autophagy and metabolism.

It was up to now not possible to maintain hematopoietic stem cells (HSC), the stem cells that generate our blood cells, in culture without driving them into differentiation. If a good protocol becomes available and works in our hands, we will move increasingly away from mouse experiments. However, it is up to now impossible to interrogate the fate of a single HSC in vitro.

Why were they not suitable?

Some experiments in mice cannot be replaced. Questions that cannot be answered with these alternatives: Does the molecular mechanism operate in the whole body as opposed to a cell in a dish? Does the identified drug cure the disease? Does a cellular pathway have an effect on the maturation of a specific cell, a process that cannot be mimicked in a dish? What is the right drug dose to use? Will the drug reach the right organ? How do cells behave in a tissue environment in which they live and what do they provide for each other?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

- based on previous experiments
- number of experiments than can be performed by the number of PILs in the lab
- breeding requirements for strains

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We calculate the number of mice for each experiment using the NC3Rs Experimental Design Assistant (EDA) online tool. We will conduct and report our experiments for publication according to ARRIVE guidelines.

We limit groups by grouping experiments, i.e test different drugs in one experiment only requiring one control group.

We are breeding mice to obtain littermates and experimental mice in a 50:50 ratio to minimise breeding of mice that are not of the right genotype.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Here are some of the measures we are taking to reduce the number of animals:

Use of pilot experiments in small number of mice, especially for those procedures, for which there is no local or international expertise.

Obtaining training from collaborators if expertise is not available

Taking into account our previous experience and those of our collaborators to determine group size

Optimising extraction methods to provide sufficient numbers of particular cell types with minimum usage of mice

Inclusion of colonoscopy will allow individual mice to be followed throughout disease development for inflammatory bowel disease. This will reduce the number of mice required to look at the disease as it progresses

Maximising use of harvested cells and tissues: setting up a shared local aged mouse colony.

Archiving of frozen tissue samples to permit analyses of novel factors without additional in vivo experiments.

Embryo but mostly sperm freezing of strains that are not currently in use.

Outsourcing to the expert: Generation of monoclonal and polyclonal antibodies will be outsourced when appropriate project authority exists

The use of genetically modified mice is a key feature of this programme. We will reduce the numbers of mice to the absolute minimum, including re-evaluating the number and genotype of breeding animals regularly. Aged mice and their littermate controls, as well as genetically modified mice and littermate controls (wild type) will be obtained from one breeding pair. Aged animals will always be aged with littermates.

13) We use CRISPR /Cas to generate genetically modified lines when possible. This is a novel technique that allows insertion or deletion of genetic material into the genome without lengthy breeding of founders obtained through conventional techniques to reduce and refine this process.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Why and how we have chosen these models and methods:

The **genetic models for autophagy** deletion are necessary as it is the only way to understand the role of autophagy in inflammation and ageing in the immune system. We have mostly chosen to delete autophagy genes in a tissue-specific way as knocking out autophagy ubiquitously (in every tissue) would cause harmful clinical effects outside the cell type of interest (such as seen in some of our models it causes anemia, myeloproliferation, or hepatomegaly). This approach avoids a harmful phenotype in tissues that we are not studying. We have also made some of the deletion models inducible, such as in adipose tissue for which it is known that deletion of autophagy in pre-adipocytes (before the fat tissue is fully differentiated into mature adipocytes) stops it from differentiating fully, preventing us from investigating autophagy's role in mature adipocytes. This refined approach ensures that we address our scientific question with the right tools,

causing less harm to the animals.

We have selected **different models of inflammation** because we don't know which site of inflammation will be most suitable to investigate during ageing. There is also not enough known in which adipose tissue immune cells reside. For the drug discovery programme some drugs might reach some sites of inflammation might better than others.

After consultation with our collaborators, we have selected the **disease models** that best model the human disease with the least side effects, i.e for osteoarthritis, rheumatoid arthritis and atherosclerosis.They were chosen to reduce number of mice (the majority of animals develop disease and therefore there is little waste) with minimal clinical signs.

To **challenge the immune system** we and other have developed tools that measure immune correlates to the named pathogens (e.g. listeria, flu, vaccinia etc). Developing these tools can take up to several years (e.g. TCR and BCR tg mice, tetramers, epitope mapping), and these are now so well established that comparison between different labs has become possible and also reproducibility within my lab is ensured. They are usually well tolerated as the immune system controls these pathogens.

We have determined doses and kinetics of **immune modulatory chemicals and biologics** in past experiments over many years of experience. For example, we have stocks of purified antibodies to deplete T cell subsets (e.g to CD4/CD8), and have figured out which LPS concentrations to minimise harmful effects and yet stimulate innate immune responses.

We have many years of experience with **bone marrow chimera** for hematology projects and they are therefore well established in my lab. They are often the only way to interrogate whether for example a manifestation of disease or cellular ageing phenotype is due to blood cells, minimise breeding of GA mice (as one is enough to generate many BM chimera) and get meaningful data. **Single cell transplantation** experiments will be rare but they are essential to understand the fate of stem cells. Any harmful effects from the irradiation procedure are now well controlled with regular monitoring.

Why can’t you use animals that are less sentient?

In order to study ageing, we have to use mice that have aged naturally. While some aging research is being done on non-vertebrates and lower vertebrates, flies and worms do not have an immune system and zebrafish does not show the same ageing features as mice or humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimise welfare costs to the animals through a rigorous model development approach and by continuously refining our protocols: through the use of pilot experiments, shortening the experiments to a minimum duration while still allowing a significant read-out and reducing dose and times of administration of substances.

We have refined the genetically modified animal breeding, as most mouse models will only lack the cellular process of autophagy in a specific subpopulation of the blood system (neutrophils, T cells, B cells), which means that these mice will not develop disease unless their immune system is challenged.

Furthermore the immune system challenge models have been carefully selected so that animals undergo minimum pain. Number of injections will be kept to a minimum, and disease duration kept as short as possible as to still allow meaningful conclusions. Painkillers will be administered where they do not interfere with the disease process.

When testing the treatment options for the disease models, we will very tightly monitor animal welfare and make sure we use the relevant biomarkers that will inform us about response to treatment and enable us to identify early humane end points to limit suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use LASA aseptic surgery guidelines. We will calculate the number of mice for each experiment using the NC3Rs Experimental Design Assistant (EDA) online tool. 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?

We are kept informed by termly welfare meetings, by our experienced NACWO and the animal house staff, by new trainees starting in the lab having attended the PIL course, and attending 3Rs conferences. We are also regularly consulting the NC3Rs website and other 3Rs websites, we are subscribed to the NC3R newsletter and changes are being implemented as information is being gained.

148. Ultrasound-mediated cavitation for enhanced drug delivery

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

Drug delivery, Ultrasound, Cancer, Cavitation, Therapy

Animal types	Life stages
Mice	adult
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 develop existing and novel ultrasound-based technologies consisting of hardware, software and ultrasound-sensitive cavitation agents (bubbles) that are used to produce mechanical forces on the micro-scale to enhance the delivery of drugs into solid tumours and we will generate proof of principle animal model data that this technology can improve the effectiveness of clinically approved drugs.

Furthermore, we aim to undertake research with industrial partners to establish the effectiveness of the technology with preclinical drug candidates.
This project will generate proof of concept data that will underpin advancement of the technology and identify drugs to be combined with it in 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?

One of the greatest challenges to effective treatment of solid cancers is the efficient delivery and diffusion of therapeutics inside and throughout the neoplastic tissue. This is due to the high intra-tumoural pressure and dense fibrous extracellular matrix (ECM) within tumours. These characteristics of tumours represent an effective barrier to drugs movement from the blood vessels into the target tumour mass despite these vessels being disorganised and discontinuous, with gaps in the vessel walls. All classes of cancer drugs are affected by these barriers but this is particularly problematic for larger biological therapeutics such as antibodies and therapeutic viruses. These large drugs poorly penetrate the tumour and so only reach a small proportion of cancer cells closest to tumour blood vessels.

This project will develop and test ultrasound-based technologies that overcome these physical barriers by using mechanical forces to pump drugs out of tumour blood vessels, driving them deeper into the solid mass of a tumour to reach and kill more of cancer cells.

In particular, we developed gas bubbles that can be made to oscillate in an ultrasound field, causing streaming of surrounding fluids and that can penetrate tumours while carrying drug in the process.

Importantly, this project will provide proof of principle data to support the application for clinical trials.

The project will also further develop the technology in terms of bubble formulations, the ultrasound hardware and software, in terms of both safety and function ahead of clinical use in man.

What outputs do you think you will see at the end of this project?

This project will result in the following outputs:

Publications on the utility of ultrasound in enhancing the drug therapy of solid tumours.

Optimization of novel bubbles for scale up production and development into a clinical product.

Identification of one or more therapeutic agents that can have enhanced efficacy when combined with ultrasound-mediated cavitation which can be taken to clinical trial.

The development of a purpose-built small animal drug delivery system for more refined evaluation of drugs in combination with ultrasound-mediated cavitation in preclinical models.

Who or what will benefit from these outputs, and how?

Data generated during this project will add to the growing body of evidence that ultrasound can be used to improve pharmacologic cancer treatment and should provide further evidence for its clinical testing.

In the longer term therefore, it is anticipated that cancer patients will benefit from the application of this technology thanks to increased treatment efficacy, leading to prolonged disease- and progression-free survival in the indications to which it will applied.

Also, the development of a small animal specific ultrasound delivery system may result in reduced animal use due to a more refined ultrasound delivery method having reduced variability that requires smaller group size for statistical robustness.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this study by actively seeking multiple collaborative partners in industry and academia with interests in diverse drug candidates of different classes and target indications for testing.

Data from our own and partnered studies will be disseminated via peer reviewed publication, presentation at conferences in the fields of therapeutic ultrasound and oncology.

Data may also be used to support subsequent regulatory filings and commercialization (e.g. clinical trial applications and patents).

Species and numbers of animals expected to be used

- Mice: 2800
- 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.

Adult mice represent a well-characterised model for the study of safety and effectiveness of drugs. Certain laboratory strains are capable of bearing tumours composed of human cancer cells without host rejection and can therefore be used to grow a model of human tumours. This offers a good model in which to test the efficacy of human-specific therapies in a whole animal system where much of the complex biology of cancer can be recapitulated. This includes the densely packed cells with poor blood supply, dense fibrous proteins and high tumour pressures that present barriers to drug penetration in human tumours. For the purposes of this project the presence of a tumour on the flanks of mice is important to enable visualisation and targeting of the tumour with the ultrasound focus without exposing the whole animal.

Adult pigs, unlike mice, offer the opportunity to test the safety and usability of patient relevant ultrasound technology in a living animal of similar sizes to a human patient. The anatomy and blood volume of a ~50 Kg pig are similar enough to an adult human's that it offers a good model for testing the capability of a developed system for imaging the internal organs, verifying the dose of ultrasound activated particles calculated for human sized animal, and testing the mechanical safety of the approach using clinically relevant equipment in advance of clinical trials in humans.

Typically, what will be done to an animal used in your project?

Mice will typically have a tumour implanted by injection of cells under the skin of a flank or,

in some cases, both flanks. Mice will have their tumours treated, under general anaesthesia, by injection of a therapeutic agent with or without the aid of an ultrasound technology targeted to the tumour. This technology consists of bubbles co-administered with the drug that are activated by ultrasound to cause a pumping effect to push the therapy out of blood vessels and deeper into the tumours. Experiments may end shortly after a single administration, where the amount of drug in the tumours with and without ultrasound pumping will be compared. Other experiments may use up to 3 rounds of this treatment and the effect on tumour sizes, or other markers of effectiveness of the drug, will be compared. Small blood samples may be taken from tails of mice during these experiments. Experiments investigating therapeutic effectiveness may last up to 120 days.

Pigs used in this project will be placed under general anaesthesia to test the ultrasound equipment. They will be infused with bubbles and the ultrasound machine will then be used to instigate the pumping mechanism and detect the acoustic signals that are characteristic of the events. Blood samples may be taken during the procedure and the animal will not be recovered from anaesthesia. The whole procedure will take up to 4 hours. Blood, post-mortem tissue samples and electronically stored data from the ultrasound machine will be used to better understand the safety profile and usability aspects of the technology.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will be implanted with cells to grow tumours under their skin. In some cases the rapid expansion of tumours under the skin of mice may, in a small proportion of animals, cause some skin redness and possible discomfort but not allowed to progress to a painful state of ulceration.

Pigs will not experience 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)?
Most mice (75%) will experience only mild severity. Up to 25% of mice may experience moderate severity. All pigs will be 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?

Whole animal models are necessary to recapitulate the complex biology of tumours and the whole body interactions with therapies and bubbles. While 3D tissue-like structures can now be reproduced in vitro they lack blood vessels, perfusion, pressure gradients, and

interactions with immune cells and are as such poor models. Furthermore, in vitro models typically lack the longevity for the purposes of this project which will investigate tumour therapies and body clearance of bubbles over the course of up to 120 days.

Which non-animal alternatives did you consider for use in this project?

Ultrasound phantoms (training aids), conventional and 3D tissue cultures, ex-vivo tissues and in-silico simulations have been considered and will be used throughout the project but the use of animals for some objectives remains necessary.

Why were they not suitable?

No non-animal alternative offers the complete system of blood perfusion of vascularised and pressured tumour and also the full metabolic capacity and immune functions necessary for full evaluation of the technology.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 therapy used in experiments under protocol 1 will be assessed in 2 phases. The first phase (drug delivery) will typically involve 3 groups of 6 mice and the second phase will typically be 5 groups of 10 mice. This requires a total of 68 mice per therapy assessed with treatable sized tumours. Typically, there will be some tumours that grow too slowly or too quickly to be included in the study and so extra mice will be implanted. Therefore, up to 100 mice will be needed per 2 phase study. We plan to conduct up to 5 studies per year totalling 500 mice per year and, over 5 years, 2500 mice.

Protocol 2 is estimated to require no more than 300 mice in total. This is based on testing 5 particle formulations for distribution in experiments with 3 groups of mice, 6 mice per group (90 mice). For lead formulations 3 longer experiments of up to 4 groups of mice in group sizes of 10 will be used (120 mice). This plan equates to 210 mice but some additional capacity has been added for unforeseen experimental needs.

A total of 20 pigs over the 5 year project is based on testing new versions of the clinical system or performance of bubbles on up to 4 occasions in each project year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order that the minimum number of mice are used the following are always considered or carried out prior to in vivo experimentation:

- In vitro tests on all bubble formulations to test biocompatibility and performance as well as in vitro test of drug and model cells to ensure relevance. This enables the elimination of certain formulations before they necessitate in vivo testing.

- Control groups will be kept to the minimum size required to provide significance as informed by pilot studies.
- Previous experiments have informed us on the group sizes needed to see a significant minimal effect.
- Can studies will be combined to share control groups?

Pig studies are minimised by only being planned as final verification of safety, usability and performance of system changes after extensive work in vitro, in silico and in mouse studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Optimum number of animals will be ensured by well performing pilot studies to give an indication of variation in data so the right number of animals can be used in later studies. By using pilot study data and relevant historical data from our own studies and setting minimum effect level we will calculate the mouse numbers required to have confidence of being able to show a result with statistical significance. This will prevent too few animals from being used to give meaningful data or more animals than necessary being used. Tissues from animals, particularly control animals, may be used across multiple experiments to limit the need for control animals in every experiment.

All pig studies will generate data for biological endpoints, usability of system, and software and algorithm performance for maximum data generation from the fewest possible 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.

Models

Mice are used for most studies on this project as they are well characterised due to the vast amount of research carried out in this species. They represent a good model for the human physiology we wish to study. Implanted tumours have a size, vascularity, intratumoural pressure and blood flow which approximates to humans. This is not the case for less sentient species such a zebra fish, flies or nematodes. Spontaneously immuno-compromised mice that do not reject human cell implants are necessary to allow testing of human specific therapies against human cell lines. Other, more standard, mouse strains with full immune competency will be used where possible in studies where mouse analogues of human therapies are possible to procure or where no tumour is necessary for the purposes of the study.

Pigs will also be used during this project. Pigs, unlike mice, offer the opportunity to test the

safety and usability of patient-relevant ultrasound technology in a living animal of similar sizes to human patients. The anatomy and blood volume of a ~50 Kg pig are similar enough to an adult humans that it offers a good model for testing the systems performance for imaging of internal organs, can be used to verify the dose of ultrasound activated bubbles calculated for human sized animal, and test the mechanical safety the approach using clinically relevant equipment in advance of clinical trials in humans.

None of the protocols will require a surgical procedure to be performed. Procedures include anaesthesia, implantation, injections, bubbles and ultrasound administration, and blood sampling.

Anaesthesia

General anaesthesia will not be undertaken more than 2 times in 24 hours or 3 times in 1 week in mice. Pigs will experience a single instance of anaesthesia (non recovery).

Implantation (mice only)

Where a tumour implantation is required we will always seek to minimise pain, distress and lasting harm by using appropriate anaesthetics. Mice will be anaesthetised for implantation of tumours and identifying microchips. Where injections/implantation result in tumour growth (in mice only) humane end-points e.g. tumour size of 1000mm^3 which is not severely restrictive to the animal will be adhered to.

Injection

Minimum injection volumes will always be used appropriate to the route of administration. Multiple injections are necessary for some experiments but analgesics will be used in the event of any injury (for example EMLA cream at site of injections if tail bruising occurs) and the animals closely monitored for health and well-being. For pigs, an intramuscular injection of sedative will always be followed by maintenance of deep anaesthesia throughout the protocol ensuring no suffering or distress and an overall mild severity.

Ultrasound administration

Mice and pigs will always be held under general anaesthesia where ultrasound is administered for the purposes of chemical restraint. No animal will receive more than 3 treatments with ultrasound and anaesthesia.

Blood sampling

Mouse blood sampling will be undertaken using best practice ensuring only the minimum useful blood volume is taken, no more than 10% in 24 h and no more than 15% of the total blood volume within 28 days. Pig blood sampling will always be taken under anaesthesia and no more than 10% of volume will be taken. Fluids will be administered to prevent hypovolemia.

General

For all procedures the training of staff will ensure the care for animal health and well-being is exemplary and there will be strong adherence to Home office and other published guidelines for animal care, training, information dispersion, record keeping.

No procedure will therefore exceed the ‘moderate’ limit.

Why can’t you use animals that are less sentient?

Mammals represent the best model for human physiology and the immune system, and they are well understood due to the vast amount of research carried out in both species included in this project. Implanted tumours in mice have a size, vascularisation, intratumoural pressure and blood flow which approximates to humans. Pigs are a length scale and blood volume similar enough to human to make good models for testing of clinically relevant whole systems. None of these arguments can be made for less sentient species such a zebra fish, fruit flies or nematodes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

A major project aim is to develop more refined animal testing equipment for administration of ultrasound effectively to mice. This will reduce time under anaesthetic and minimise animal stress.

Animal monitoring will be increased in the event of any harms identified following procedures and, where possible, refinements made to procedures to limit any chance of recurrence of the harm.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Many sources of information are available to ensure experiments are the most refined. These include:

- LASA guidelines on best practice
- NC3Rs guidance such as procedure with Care and guidance on blood sampling
- Research Animal Training Resource
- ASRU guidance on aseptic technique

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am subscribed to the NC3Rs news letter and receive regular updates and Operational Newsletters issued by the Animals in Science Regulation Unit.

Team members will attend relevant local events such as organised 3Rs research days.

Implementation of any changes will be done through consultation with vets, NACWOs and NTCO to identify the need for any licence authority changes before any change to a procedure.

149. Investigating mechanisms of immune activation for improved vaccination and understanding immune-mediated 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
  - 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

vaccines, adjuvants, antibodies, autoimmunity, allergy

Animal types	Life stages
Mice	adult, neonate, juvenile, 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 twofold: i) to better understand how to optimally activate the immune system to deliver improved vaccines, ii) to better understand how to prevent unwanted activation of the immune system so as to reduce immune-mediated diseases such as allergy and autoimmunity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits

that accrue after the project has finished.

Why is it important to undertake this work?

Aim 1. The immune system has evolved to control and eliminate infectious agents such as viruses and bacteria and to counter cancer, and can be harnessed by vaccination to provide immunity against these infectious agents and potentially cancer. Despite a great deal of success with many protective vaccines however, others are more challenging, such as that for the human immunodeficiency virus type-1 (HIV-1) and against cancer. Understanding how to optimally activate immune responses is central to improving the efficacy of vaccines and reducing their side-effects.

Aim 2. Unfortunately, when inappropriately activated, the immune system can cause disease, such as allergy and autoimmunity. Understanding how to reduce and/or prevent unwanted immune responses is important for understanding and mitigating immune-mediated disease.

The immune system is a double-edged sword and these two aims are two sides of the same coin, therefore integrating their study into a single project is logical and efficient.

What outputs do you think you will see at the end of this project?

There are two objectives, and the outputs will be specific to the objectives.

Objective 1. We expect to develop new vaccination approaches based upon combinations of novel modified antigens with immune activating agents called adjuvants. These vaccine approaches will have specific relevance to HIV-1 but also inform vaccine design against other pathogens and cancer.

Output 1: New information for dissemination and publications

Output 2: We intend that at least one new vaccine concept should move into a clinical trial within the lifetime of the new license.

Objective 2. Our major output will be increased understanding of molecular and cellular mechanisms underlying the triggers of immune dysfunction in autoimmunity and allergy.

Output 1: New information for dissemination and publications.

Output 2: Promote new lifestyle-based approaches, based upon reduction of endogenous (inflammation) and exogenous (dietary, industrial) sources.

Output 3: Suggest new approaches to therapeutic intervention such as drugs to reduce inflammatory activation of the immune system.

Who or what will benefit from these outputs, and how?

Objective 1. HIV-1 currently infects about 38 million people, and we have no effective vaccine. Similarly, it is estimated that one in 3 people will succumb to cancer, but despite conceptual promise, vaccination against cancer has not been successfully realised.

Benefit 1: Progress made under this proposed project towards new approaches to vaccine

development will build a platform of knowledge that will allow ourselves and others to begin to translate this over the next 5 years into clinically-relevant vaccines to protect against HIV-1, other hard to vaccinate infectious diseases, and potentially cancer.

Benefit 2: Provide proof of concept for anti-cancer vaccination and promote a novel approach to this problem.

Benefit 3: Cellular and molecular mechanistic insight to be communicated to the academic and industrial communities.

Objective 2. To make further progress in defining molecular changes that lead to activation of unwanted immune responses that underlie immune-mediated allergic and autoimmune diseases.

Benefit 1: Success in identifying the precise molecular and cellular triggers of the mechanistically- linked diseases of autoimmunity and allergy would lead in time to policy changes driving lifestyle changes to reduce the generation and impact of endogenous and exogenous aldehydes.

Benefit 2: The development of new more targeted drugs to combat these diseases. This would directly benefit millions who are affected by these increasingly prevalent diseases.

Benefit 3: Cellular and molecular mechanistic insight to be communicated to the academic and industry communities.

How will you look to maximise the outputs of this work?

- Our research is multidisciplinary (immunology, cell and molecular biology, chemistry) and has relied strongly on national and international academic collaborations, and we will enthusiastically continue this.
- We have established and mutually benefitted from industry collaborations that aim to improve vaccine efficacy and design new vaccines, and will continue this in the new period.
- New knowledge, including unsuccessful approaches, will be disseminated by the usual channels including to academics and industry via preprint servers and peer-reviewed publications in open access journals, and presentation at national and international meetings.
- Knowledge will be disseminated to the public by public websites, media outlets, public lectures and social media.

Species and numbers of animals expected to be used

- Mice: 4000 wild type and 1000 genetically altered

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 vertebrate group in the evolutionary tree for which suitable models of vaccine-relevant immune responses and immune-mediated disease are available, and so work cannot be done using lower vertebrates or invertebrates.
- We will carry out work in inbred mice since they are genetically identical and this allows meaningful group sizing for statistical power whilst helping to minimise animal numbers.
- Because of the numerous different immunological tools and large data sets already available, mice can be used for detailed in vitro and in vivo characterisation, which is not generally possible in other small mammals.
- Genetically altered (GA) mice provide a unique opportunity to study the role of particular genes, molecules and pathways in the induction of beneficial and deleterious immune responses. Such a range of genetic alterations are not available in other mammalian species.
- Adult mice will be used to model a large segment of humanity with active healthy immune systems.

Typically, what will be done to an animal used in your project?

- Typically, wild type (WT) and/or GA mice will have a small amount of blood sampled from a tail vein, then an immune modulating substance such as a cell-depleting antibody or an innate immune modulating substance previously shown by us or others or described in the literature as harmless will be administered with frequencies and dosing as below.
- A protein, carbohydrate or glycoprotein antigen optionally formulated with a non-toxic adjuvant or immune-modulatory substance previously shown by us or others or described in the literature as harmless will be administered. Administration will be via one of a range of routes with the frequencies and dosing as below. Administration will be by the most refined routes and frequencies required to achieve our scientific objectives.
- A typical experiment would last for 3 months. At the end of the experiment mice will be killed by a Schedule 1 method usually associated with exsanguination under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals are not expected to suffer any pain beyond transient mild pain associated with blood sampling (30 seconds) and associated with injection or administration of small volumes of harmless substances (10 seconds). However, over the longer version of protocol 1 in which there are multiple administration and sampling steps (~20% of experience), animals may accumulate sufficient interventions for this to be classified as 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 is moderate for protocol 1 for ~20% of animals and mild for the remaining ~80%, and mild for protocol 2.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

- It is not possible to replicate the complexity or dynamics of host immune system interactions in vitro. For example, there is no physiologically-relevant in vitro system to mimic vaccination by eliciting specific antibody and T cell responses leading to production of neutralising antibodies required for successful vaccination. Similarly, efficacy and safety profiles of vaccine adjuvants and other immune modifiers cannot be determined in vitro, but must be determined empirically in vivo.
- The model systems we will use to analyse unwanted immune responses will require the full complexity of the immune system to pinpoint how all components of the immune system interact to trigger autoimmune and/or allergic antibody and T cell responses.
- Models of priming for immunological disease that we propose for autoimmunity, allergy and hypersensitivity are considered to be closely representative of the human disorders, are well- established, and safe from overt clinical symptoms.
- Because of the numerous different immunological tools available, mice can be used for detailed characterisation, which is not possible in other organisms.
- GA mice provide a unique opportunity to study the role of particular genes, molecules and pathways in the induction of beneficial and deleterious immune responses.

Which non-animal alternatives did you consider for use in this project?

- We have carried out T cell analyses of intrinsically-adjuvanted antigens through in vitro antigen processing using immune activating cells called dendritic cells isolated from a small number of mice and presentation to immortalised T cell hybridomas as an alternative approach to in vivo testing in mice. However, we acknowledge that these assays do not recapitulate the in vivo phenotype.
- In future, we will test in vitro models of reconstituted tissue culture mini-organs (eg. germinal centres) for their ability to respond to immunogens in our systems.
- We are committed to moving towards use of human immune cells for these studies, and are experimenting with ex-vivo analysis of B and T cell responses derived from human peripheral blood. However, these will be confirmatory of mouse experimentation rather than replacement, as they lack the sophistication and complexity of the intact immune system.

Why were they not suitable?

- In vitro antigen processing using dendritic cells isolated from a small number of mice and presentation to immortalised T cell hybridomas was tested as an alternative approach to in vivo testing in mice, but we realised that these assays did not recapitulate the in vivo phenotype. We therefore use these assays as a parallel approach to add information, but not as a substitute to in vivo experimentation.

- At present use of isolated human B and T cells does not provide sufficient antigen-specific precursors to yield a useable readout, however we continue to work on this so as to optimise experimental conditions.
- Reconstituted tissue culture germinal centres are generally acknowledged to be not yet sufficiently representative of the in vivo complexity so as to be able to fully recapitulate an adaptive immune response in vitro.
- Whilst human tissues are helpful to confirm phenotypes once mechanisms have been defined, they are less tractable for dissecting mechanisms, as they lack the required in vivo complexity.
- Whilst it is possible to carry out genetic manipulation of immortalised leukocyte cells lines to probe mechanism, it is well accepted that these lines do not fully physiologically representative of the intact primary immune system. Genetic manipulation of primary cells and tissues is far more technically difficult and causes unwanted side effects that are difficult to control for, such as activation of the innate 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 is estimated upon previous experiments we have undertaken for these projects.
- The estimated number of experiments required to address the experimental objectives.
- The number of PILs in the laboratory that can carry our mouse experiments.
- The breeding requirements and availability of GA strains.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- We will calculate the number of mice for each experiment using the NC3Rs Experimental Design Assistant (EDA) online tool.
- We limit groups by block grouping experiments, for example testing different variables in one experiment only requiring one control group.
- Male and female mice from the same litters will be used where possible.
- GA mice are bred to obtain litter mates and experimental mice in a 1 : 1 ratio to minimise breeding of mice that are not of the right genotype.
- Heterozygote GA mice will be used as controls for homozygotes where possible.
- Experiments will be conducted and reported for publication according to 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?

- Use of small numbers of mice in pilot experiments, particularly for experiments where there is no precedent in the literature or locally.
- Where possible source GA animals from external sources to reduce breeding.

- Optimum group size will be informed by the relevant literature, by previous work in the lab or locally, and/or by pilot experiments.
- Obtaining training in specific techniques from collaborators if expertise is not available to reduce failed experimental outcomes.
- Optimising extraction methods to provide sufficient numbers of particular cell types with minimum usage of mice.
- Maximising use of harvested cells and tissues for multiple experiments across the different objectives.
- Archiving of frozen tissue samples at experiment termination to permit further analyses without additional in vivo experiments.
- Sperm and/or embryo freezing of strains that are not currently in use.
- Use of specific GA mouse strains will be reviewed frequently to ensure only those currently required 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.

- Immune activating agents may, in some cases (such as Freund's Complete Adjuvant), cause long- term inflammation and associated tissue damage. Such agents with known deleterious effects have been replaced by other safer agents that we have identified over the previous PPL periods, and new agents which we continue to explore.
- Over the past decade we have optimised doses and kinetics of immune modulatory chemicals, adjuvants and biologics so as to deliver the desired response with minimal adverse effects.
- Our antigens are highly purified in-house, and we go to a great deal of trouble to reduce or eliminate contaminating substances that might influence immunological outcomes in all samples destined for in vivo use.
- For objective 2, mouse strains that are genetically and phenotypically sensitive to allergic or hypersensitive priming will not be used for our models to avoid any clinical signs such as allergy, anaphylaxis or overt autoimmunity.
- Although our models for allergy and other immune diseases are designed to avoid clinical signs, they will be further refined over the course of this license to reduce any potential for animal suffering by optimising doses and timing of administrations.
- We have selected different routes of administration of immune activating agents firstly to probe the adaptive immune response to models of vaccination at different sites and via different routes to induce local or systemic immunity (objective 1), and secondly because we would like to induce unwanted immunity (objective 2) via different sites to mimic the human situation - for example oral, intradermal and transdermal for food allergy and intolerance, systemic for autoimmunity.

Why can’t you use animals that are less sentient?

Analysis of mechanisms underlying how the immune system is activated in vaccine design (objective 1) and immune pathology (objective 2) cannot meaningfully be carried out in non-mammalian species as their immune systems are too divergent from man, and invertebrates do not have an adaptive immune system that contains T cells and B cells that make antibodies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimise welfare harm to the animals through rigorous and frequent refinement of our protocols including:

- The use of pilot experiments.
- Shortening experimental timeframes to a minimum duration whilst conserving experimental outputs.
- Reducing dose and times of administration of substances where possible.
- Continuously reviewing the literature for novel immune modifiers and vaccine adjuvants that may be used to replace the current ones.
- Refinements in monitoring to detect any mild but noticeable physical, behavioral or postural changes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- We will calculate the number of mice for each experiment using the NC3Rs Experimental Design Assistant (EDA) online tool.
- 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?

- Termly compulsory departmental animal welfare meetings.
- Sharing new PPL-relevant information at lab meetings.
- Notifications from our experienced NACWO, Vets and animal house staff.
- Attendance of new trainees in the lab at PIL courses.
- Attending 3Rs conferences.
- Lab members regularly consult the NC3Rs website and other 3Rs websites and we subscribe to the NC3R newsletter.

150. Investigating the immune response in liver disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immunology, Liver disease, Vaccines, Therapy

Animal types	Life stages
Mice	adult, juvenile, 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 immune system relevant to liver disease, and how the immune system responds to viruses and vaccines relevant to liver disease, and to use this information to design improved vaccines and 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?

Viral hepatitis is a global health burden that afflicts approximately 325 million people worldwide (WHO Viral Hepatitis Report 2017). Despite a licensed preventative vaccine against hepatitis B virus (HBV) and drugs for hepatitis C virus (HCV), the overall number of infections has continued to rise over the past several years resulting in approximately 1.5 million new infections per year. Therefore, the World Health Organisation (WHO) has identified viral hepatitis as a priority area with the aim of elimination by 2030. Central to this elimination strategy is the development of a prophylactic HCV vaccine and new

curative HBV therapies. In order to better develop vaccines and therapies against viral hepatitis, the immune response to new vaccines and therapies must be investigated. Therefore, the investigation question is a physiological one that requires the analysis of the immune system in its entirety, i.e. in a living organism, to determine the best candidates to advance into human trials.

What outputs do you think you will see at the end of this project?

By the end of this project, we aim to have determined the immunogenicity and effectiveness of vaccines to prevent and treat viral hepatitis by measuring immune responses and defining the protective capacity of these responses in animal models. Therefore, the main output will be the identification of at least eight optimal HCV and HBV vaccine candidates that we may progress through human clinical trials as part of the licensure process. It is expected this work will comprise a number of high impact publications.

Who or what will benefit from these outputs, and how?

Currently, there is no licenced HCV vaccine to control increasing global infection rates, and there are limited effective therapies for HBV for which there is approximately 257 million HBV-infected people who could benefit. Within the timescale of the project, we hope to have answered key questions about liver immunity, which may support the development of viral hepatitis vaccines. The proposed work represents an important leap forward in the development of viral hepatitis vaccines and therapies as the outputs constitute both long-term and short-term benefits to patients and researchers. For example, the results of this project will inform human studies as part of the licensure process. In addition, the research community will also benefit from knowledge obtained.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, we have formed collaborations with leading experts in specific aspects of viral hepatitis research in order to advance the work, disseminate new knowledge and expertise, and promote our findings. We intend to publish and present our findings at key international conferences including the annual HCV and HBV conferences and the European Association for the Study of the Liver (EASL) International Congress. We will publish our findings in high impact journals.

Through our collaborations, we have already secured numerous grants, shared resources, and engaged in scientific discourse to advance the work to this stage. Throughout the project, we will utilise both our laboratories to investigate the research questions. We will also secure the remaining intellectual property (several patents filed already both in the U.K. and elsewhere) in order to seek commercial commitment from external funders including venture capitalists to advance the vaccines into human clinical trials.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The research question being asked is a physiological one, which requires analysis of an immune response in a living organism to adequately assess vaccines and therapies. Scientifically, immunisation and challenge of mice with a virus is standard way to address questions about the immune system. The adult mouse is a very well-defined model for immunology experiments and the models proposed (immunisation with vaccines) is highly reproducible, enabling us to ask research questions that are highly relevant to a human clinical vaccination setting. Both inbred and outbred strains of mice will be used to ensure reproducibility and representation across different genetic backgrounds, without producing too much variation between mice in the same experiment group caused by individual genetic diversity.

Furthermore, animal models of viral hepatitis (RHV and AAV-HBV) to assess vaccine/therapeutic efficacy have been specifically developed for mice. Until recently, lack of robust, readily available, immunocompetent mouse models to test vaccines/therapeutics has been a significant bottleneck in the field. Therefore, these newly developed mouse models will be ideal for answering specific questions about vaccine and therapeutic efficacy.

Neonatal, juvenile, adult and pregnant mice are required to control husbandry and breeding methods for genetically modified mice.

Typically, what will be done to an animal used in your project?

Typically, a mouse receive an injection of a vaccine one to two times in the muscle (IM) or vein (IV), followed by several blood tests over an 8-week period, all animals will be humanely killed before one year of age.

In addition to the typical vaccine studies above, animals may also be challenged with relevant infectious agent (e.g. RHV and AAV-HBV)

What are the expected impacts and/or adverse effects for the animals during your project?

Vaccine/therapeutic administration: Animals will typically show no clinical signs in response to vaccine/therapeutics/challenge agents. A very small number of mice, may experience short term discomfort (less than 72 hours) following vaccination.

Challenge/infection agent administration: Clinical signs are expected to generally be mild. Injected compounds are expected to be non-toxic or have limited toxicity at the doses used. Injections are not expected to cause more than transient discomfort.

For both the RHV challenge model and the AAV-HBV infection model, adverse effects have not been reported by other groups working with this infection model. However, following the 1-3 days after inoculation, possible adverse effects may include those typical of any viral infection or viral hepatitis. For the very small number of animals that do exhibit these clinical signs, we expect effects to not exceed 72 hours.

Chemical agent administration: A very small number of mice will be administered chemical agents such as tamoxifen or doxycycline. For these animals, weight loss of 5-

10% is typically transient and recovers within 72 hours.

No adverse effects are expected from administration of extracellular/intracellular (expect tamoxifen) or blood sampling steps.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

95% mild

5% moderate

What will happen to animals at the end of this project?

- All animals will be humanely killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Despite efforts to carry out replacement strategies using organ cultures, not all cell types involved in vivo can be successfully grown in vitro, nor can the complex interactions of multiple cell types be fully studied. Therefore the immune responses cannot yet be fully replicatedoutside of a living organism. Consequently, animal models are still required to study the mechanisms of immune responses during vaccination and infection.

Which non-animal alternatives did you consider for use in this project?

Whilst animals may not be wholly replaced to address the overarching research questions, the use of human data, in vitro, and in silico methods may also contribute to the programme of research.

Human data: The lab works with human studies including analysis of Phase 1 clinical trials. These trials have focused on the development of adenoviral vectors for prevention and therapy of hepatitis C Virus and for protection against hepatitis B virus (HBV). We have ongoing studies of HCV and HBV in the lab. These data have been used to plan better experiments in the mouse model, which can then be used to address questions most relevant to the clinical setting.

In vitro methods: We are developing in vitro models to study some of the most simple elements of virus persistence in cell culture. For example, we have set up infectious model systems for HBV and HCV where intracellular responses can be assessed. If we have indications from these cell culture models which could lead to replacement of the planned in vivo work, we would take advantage of this.

In silico methods: We have used and will continue to use in silico methods for the

optimisation of viral constructs prior to in vivo experiments, thereby replacing the use of some animals.

Why were they not suitable?

The experiments planned are only those where we cannot achieve the same objectives by analysis of human subjects.

Such ex vivo experiments cannot address the immunogenicity of a range of vaccines - we can only test very few vaccines in humans. To define which of the potential approaches might be best (and safest) we still require an intact and flexible in vivo system that allows us to assess the efficacy of such induced responses against challenge.

While the use of in silico methods may inform vaccine optimisation and in vitro assays can be used to confirm key findings of this project, the research question being asked is a physiological one, which requires analysis of an immune response in vivo.

It is not possible to address the importance of specific factors in vitro and capture the full complexity of the immune system and its protective capacity against liver disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals based on previous usage over a period of time. For each individual experiment, numbers used are based on power analyses to attain the correct number of mice per group to assess our research question (typically n=5-10 per group for immunogenicity). Based on previous experiment design, we typically use between 10-40 mice per experiment as multiple experiment groups comprise of different vaccine designs, immunisation regimens, and/or necessary controls.

In analyses of immune responses as described in the key experimental protocols, we typically find small standard deviations (measures of how variable the readings are). In these experiments we are also looking for large effects (e.g. 5-fold), but also those which distribute over time, which increases the power of detection. In a typical experiment, if the vaccine induced T cell response is 2%, with a standard deviation of 0.3, we can detect a 50% reduction in response in the experimental group (with similar standard deviation), with $p=0.001$ (a p value less than 0.05 is generally regarded as significant in such studies). If the standard deviation were increased to 0.66 (range 1.2-2.8 for the control group and 0.2-1.8 in the experimental group), we can still detect a response at $p<0.05$. Thus, for experiments of this kind 5-10 per group provides appropriate sensitivity to detect moderate effects even at a single time point.

The proposed work in this licence includes the assessment of vaccines in transgenic mice such as HLA-A*0201 humanised mice and KO mouse strains such as IFN-/- and TNFa -/-, etc., over multiple grants. Therefore, the 3000-mouse limit for breeding reflects the work

requirements of our current and imminent grant-funded work with excess for future grants that we will secure within five years. For all of the work in both these grants and future grants, we expect that half of the animals will not be used for experiments because 1) they will be used as breeders or 2) they are transgenic offspring with an undesired genotype or gender. The remaining number will be used for experiments. The proposed animal number limit is also in line with our previous experience of breeding multiple mouse strains for assessment of vaccines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In most cases, repeated longitudinal measurement of the immune responses over a period of time will keep numbers to a minimum, instead of harvesting groups of mice at each time point. This confers the advantage of not only keeping animal numbers down but also enables us to monitor changes in the immune response in each mouse over time, particularly in the context of vaccine-induced protection against challenge.

We have used the NC3R Experimental Design Assistant tool to assist the design of our animal experiments under a previous licence. We have also used pilot data from this licence to calculate power analyses to ensure the correct number of animals is used without sacrificing the power to address the research hypothesis. In addition, we will use blinding and randomisation where possible to further ensure the generation of robust scientific data with minimal 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?

For breeding KO mice, we will use the MCMS database as a tool to optimise breeding efficiency. efforts will always be made for those members of staff working within this project to where possible share or combine usage of mice.
Furthermore, all vaccines have been optimally designed using computer modelling prior to production and testing in mice. Pilot studies for vaccine immune assessment have also been undertaken to ensure appropriate group numbers are used to rigorously test scientific questions while keeping the number of mice to a minimum. We routinely use different tissues from a single animal at the end of the study instead of using different animals for different tissues. The approach reduces the overall number of animals used and enriches the downstream dataset by allowing for comparisons between different tissues in the same animal (e.g. the correlation of the T cell response in the blood versus the liver, or the correlation of the T and B cell response in the lymph node versus the antibody response in the blood).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 complex interaction of immune cells and tissues dictates the need for using model organisms.

Mice have a relatively short lifespan and are also considered the least sentient species available for this type of analysis of immune correlates of protection for many human pathogens**.**

Mice are well-defined model for immunologic experiments. The proposed methods using viral vectors is highly reproducible and therefore can be used more efficiently and with the maximum refinement and reduction. We will also perform a limited number of experiments in mice to test the efficacy of candidate vaccines against HCV and HBV, using the newly developed models: mouse-adapted RHV challenge model and the AAV-HBV challenge model. Both challenge models do not cause distress to the animal as disease is asymptomatic and in the case of RHV, self-resolving after 5 weeks, causing no harm to the animal. For HBV, chronic infection is not associated with liver damage or associated symptoms.

In particular we will focus on the use of the adenoviral vector model, which has the most limited impact on the animal's welfare and provides clinically relevant data.

Why can't you use animals that are less sentient?

The mouse immune system has been shown over time to be a good model to use for human immunity, especially in the context of vaccines. Only mammalian immune systems contain all the relevant components we need to analyse and the well-defined structural composition (lymph nodes and organs) makes this a very accurate system to define the totality of the immune response of the animal. The experiments require days or weeks for the immune responses to develop and require an intact adult animal for completion.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise welfare costs, we will work closely with the animal care team to provide the best environment for the mice, and ensure appropriate monitoring is in place to effectively assess their welfare status. This monitoring will be tailored for the experiment and will be reviewed regularly to ensure it is suitable and allows the team to limit any harm to the animals.

For vaccine/therapeutic/infection/challenge agents: each agent will be administered to the animal by the most refined route according to scientific need and at an appropriate dosage to limit adverse effects, if known.

For new dosing regimens (e.g. challenge agents, vaccine, therapeutics), we will perform a small dose escalation study (n=3-5 sample size based on previous experience) which includes a single injection with the agent (staff blinded to the agent and dosage administered) at low, medium, and high dosage to ensure that there is some measurable effect of the agent at the lowest possible dose, typically over a 2- 3 week period for vaccines/therapeutics and a 5-10 week period for challenge agents. Only one dose regimen will be tested per group of mice.

After agent administration, animals will be monitored for clinical signs of expected adverse

effects including daily observation of any change from pre-administration behaviour and weighing for up to 3 days post-administration.

For administration of antibodies or sera, the intravenous route will be used for the first administration of previously untested antibody to minimise the risk of anaphylaxis.

When feasible we will seek to replace repeated injections (e.g. daily) by other such methods such as the implantation of a minipump to reduce stress and discomfort of caused by multiple injections.

Chemical agents: These will be administered to the animal by the most refined route according to scientific need. Where more than one substance is administered, both will have been previously been tested as single agents or their tolerability will already be known. Intraperitoneal (i.p.) administration can be used as an alternative to oral gavage.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

A number of approaches will be used throughout the project to minimise adverse effects. Published guidelines for best practice will be followed: eg (i) Wells et al, Assessing the welfare of genetically altered mice. Laboratory Animals 40(2), 111-114 2006; (ii) Laboratory Animal Science Association Good Practice Guidelines Series 1/Issue 1) October 1998. Administration of Substances (Rat, Mouse, Guinea Pig, Rabbit) and Collection of Blood Samples (Rat, Mouse, Guinea Pig, Rabbit).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All PIL holders and I will sign up to the 3Rs mailing lists (if not already) including the University's 3Rs newsletter to receive important updates. As a lab group, we have established bimonthly meetings to present data and discuss relevant updates regarding the animal work, including NC3R updates, and online 3R resources. There are also termly meetings for all PPL and PIL holders in the University where information on the 3Rs is often disseminated. We have also implemented the NC3Rs online experiment design assistant tool into our existing workflow.

We also use the handbook, Festing MFW and Altman DG, 2002 ILAR J; Festing MFW, Overend P, Gaines Das R, Cortina Borja M and Berdoy M, The Design of Animal Experiments (2002) Laboratory Animals Ltd., London, to inform experimental design.

151. Experimental Interventions in Large Animals Under Terminal Anaesthesia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

pigs, small ruminants, translational research models, terminal anaesthesia

Animal types	Life stages
Pigs	juvenile, adult
Sheep	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 overall aim of this project is to identify the translational research potential of biomedical ideas, hypotheses, prototypic technologies and pharmacologic agents through small-scale "proof-of-concept" studies conducted on large animal models under terminal anaesthesia, before larger scale studies authorised under separate license are designed and funded. A parallel and complementary aim is to optimize the use and welfare of large animal species (and minimize the numbers required) in studies where a key focus is to minimizing the interval between the inception of medical ideas and their deployment into the human 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?

It is becoming increasingly recognized that biomedical studies conducted on traditional laboratory animal species, e.g., mice, may yield results that delay or even prevent medical innovations, e.g., drugs, being used in potential human beneficiaries. Such delays are also associated with the waste of animals and scientific resources including research funding. Using carefully chosen size-relevant (large) animal models offers numerous advantages over smaller species and may expedite the translation of medical advances into clinical medical practice. Studies performed under expertly- managed terminal anaesthesia will be optimally refined, and may require the use of fewer animals to achieve study objectives

What outputs do you think you will see at the end of this project?

This project's general aim is to improve the efficiency and accelerate the progress of translational projects, i.e., the development of diagnostic and therapeutic tests, equipment, procedures and ideas and their subsequent introduction into medical (and possibly veterinary) clinical practice. It will achieve this in large part by capitalizing on the skills of specialist veterinary anaesthetists whose ability to maintain stable physiological, biochemical and haematological conditions within narrow ranges and for prolonged periods (up to 96 hours) will ensure the collection of maximum volumes of high-quality data from each animal used, leading to a reduction in the overall animal requirement. Therefore, the primary immediate outputs are quantitative data and qualitative information that will inform decision- making in the translational research process, e.g., i) to end all study; ii) to continue pilot work after revising current methods, or; iii) to apply for funds for larger-scaled studies under separate license.
However, an important secondary immediate output, is - where required - the publication of pilot research findings describing the unsuitability, or otherwise of experimental methods, including the animal model itself.

Mid-term outputs would include - confidentiality agreements permitting - the publication and presentation of new data providing support for the initiation of clinical trials in the target population.

Secondary mid-term outputs will also include the publication of details providing new and, or critical information on the experimental methods used.

The different protocols within the project have previously (under the applicant's preceding license) encouraged synergistic interaction between different, ostensibly unrelated academic specialists, e.g., mechanical engineers and medical clinicians, which in turn has led to the generation of new ideas and further grant applications.

The constant emphasis on experimental refinement will result in the development, publication and dissemination of techniques improving laboratory animal use, care and welfare.

Who or what will benefit from these outputs, and how?

General.
Conducting pilot studies under prolonged terminal anaesthesia will provide initial information on the suitability of currently described experimental techniques, including the large animal models themselves. This will later lead to the more efficient investigation of diseases of importance to human beings and, perhaps, other animals. This will provide mid-term financial benefits for funding bodies and researchers. Animal use will also be reduced. In the long-term, biomedical advances will benefit both human and veterinary

clinicians and patients.

This project will use pigs and small ruminants to test new and improved therapeutic strategies (including technologies and pharmacologic methods) to diagnose and treat illnesses in human beings. In addition, the meticulous management of the animals which will involve the collection of an extensive range of physiological, biochemical, haematological and anatomical (imaging) data, will be collected, archived, and disseminated in order to improve the management of animals in this and future studies.

The long-term primary and secondary purpose of these outputs is to improve human health while refining animal experiments by improving laboratory animal care. A long-term secondary benefit may be an improvement in animal health.

Benefits 1) The Development of Capsular Technologies for Gastro-intestinal Disease Management.

The development of autonomous swallowed capsules engineered to identify, localise and subsequently treat gastro-intestinal conditions will provide a less invasive and more acceptable option than are currently available, e.g., colonoscopy, surgery and systemic chemotherapy - and on a far greater scale than is currently possible.

Benefits 2) The Use of Porcine Acute Respiratory Distress Syndrome Models in Developing Clinical Management Strategies

Inducing acute respiratory distress syndrome (ARDS) in terminally anaesthetized pigs by oleic acid infusion (or other methods) will facilitate the evaluation of diagnostic methods, e.g., optical molecular imaging [PROTEUS] and putative therapies, e.g., N2, leading to an improved ability to manage this condition - a major cause of death in medical intensive care units.
Benefits 3) The Development of Miniaturised Sensor Electrodes in the Management of Gastro- intestinal Anastomotic Leakage.

The implantation of appropriately calibrated miniaturised sensors in proximity to surgical wounds, e.g. colo-rectal anastomoses, may allow the early detection of post-operative wound breakdown and its adverse consequences, i.e., bowel leakage, peritonitis and death.

Benefits 4) The Use of Ovine Pulmonary Adenomatosis (OPA) Models for Evaluating New Methods of Lung Cancer Management

Methods improving the early diagnoses of lung cancer will improve the prognosis in, and survival time of human cancer patients. New techniques for the localised rather than the systemic administration of chemotherapeutic drugs will reduce the incidence and severity of the unpleasant side-effects associated with cancer treatment.
Benefits 5) The Use of Porcine Renal Ischaemia-reperfusion Injury Models.

An advanced and refined large animal model of acute kidney injury will be used to undertake early evaluation of drugs with potential benefit to the large number of people who develop this condition annually. The model offers several benefits over previous models and those in other species. Its features could allow identification of new markers of kidney injury that would enable earlier diagnosis of kidney injury before irreversible damage has occurred. It may also allow a greater understanding of the disease process itself.

Benefits 6) The Use of Piglets to Study Hypoxic / Ischaemic Tissue Injury During Childbirth..

Current methods for detecting the wellbeing of babies during childbirth do not always identify situations threatening the babies' life. Testing new approaches and methods that continuously monitor oxygenation and other metabolites in anaesthetized piglets - and their adverse consequences, e.g., raised intracranial pressure - will accelerate the development of devices for assessing a baby's wellbeing throughout labour and reduce the risk of lethal consequences.

Benefits 7) Electroanatomic Studies of the Large Animal Heart

Atrial fibrillation (AF) - a debilitating, and potentially fatal condition of increasing world-wide importance which is most commonly managed by radio-frequency ablation (RFA) - is poorly understood. An increased understanding of factors affecting atrial conduction pathways together with a more accurate method of measuring atrial wall thickness will contribute to a better understanding of AF, whilst improving the success rate of, and diminishing the incidence of side-effects associated with RFA. The development of electroanatomic mapping technologies using large animal models, with or without AF, will expedite their translation into medical practice.

Benefits 8) Safety and efficacy testing of new imaging/diagnostic & tissue-sensing technologies.

There is a need for technologies that allow surgeons and physicians to rapidly identify and, or quantify tissue pathologies and, or trauma which will expedite decision-making during surgery and, or situations where delay penalties exponentiate. For example, the ability of cancer surgeons to ensure that all cancerous tissue has been removed during surgery (through purposed imaging or haptic sensing technologies) would reduce the risk of metastatic spread and the need for re-operation, and ultimately prolong and increase the human patient's duration and quality of life, respectively. In particular , the ability to quantify the physical characteristics of tissue, e.g. impressionability - or other qualities currently achieved by a digital "poke" or manual palpation - would reduce inter-individual variations between those applying the test and help focus therapeutic decision-making. Developing safe and effective imaging and, or quantitative tissue-sensing technologies that are safe, accurate and readily applied during surgery, or at other bedsides, would make these objectives achievable.

How will you look to maximise the outputs of this work?
The licensee will ensure that all notable study findings - particularly those pertaining to experimental refinement - will be incorporated either into the main study report, or as an independent publication. The publication of critical re-evaluations (and condemnations) of unsuccessful methods including animal models, will be prioritized

Species and numbers of animals expected to be used

- Pigs: Approximately 150 (or 120) over 5 years
- Sheep: Approximately 20 over 5 years
- Minipigs: Unknown; estimate 30 in lieu of pigs enumerated above

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Pigs of varying ages and size are established and accepted models for the study of conditions affecting the human gastro-intestinal tract, liver, kidneys, heart and blood vessels, lungs and skin because these organs are similar in size, anatomy and function to those from humans. Experiences gained under previous license have largely confirmed this widely-held view.

Ovine Pulmonary Adenomatosis is endemic amongst the Scottish sheep flock and a common cause for culling. Using affected animals in lung cancer research is an efficient and ethically defensible application of a natural model.

Similarities in the extent of human, porcine and ovine gyrencephalisation offer specific advantages over laboratory rodents in the development of brain imaging and surgical methods.

Typically, what will be done to an animal used in your project?

All animals studied under this PPL will be terminally anaesthetised. Most will be acquired from local flocks and herds, whose health status is well-known and with whom the PPL holder has had a long (> 30 years) relationship. Transit times will typically be brief, i.e., 10 - 30 minutes. When this is so, the delivered animals will be examined and those in good health will be sedated immediately upon arrival and anaesthetized shortly thereafter. For animals undergoing more prolonged journeys, i.e., 1 - 3 hours, appropriate periods and conditions of acclimatization will be imposed. In studies, involving prolonged transit, and, or when the HOI / NVS stipulate, animals will be transported and inducted to the study in pairs. In the majority of projects, the animal's most noxious experience (after transport) will be a single IM (pigs) or IV (small ruminants) injection of pre-anaesthetic medication. (In two protocols, animals may also be given substances, and, or blood sampled). Thereafter, anaesthesia will be induced and maintained to the highest possible standards - being managed by Specialists recognised by the Royal College of Veterinary Surgeons (RCVS) and the European College of Veterinary Anaesthesia and Analgesia (ECVAA) or their trainees. The extent and methods used to achieve physiological instrumentation will be commensurate with study (protocol) requirements and duration of the study but will typically involve invasive vascular access and urinary bladder catheterisation.

Anaesthetized animals are likely to then undergo additional surgery, with, or without device implantation, imaging, and, or drug or substance administration, according to the studies needs. Animals will remain anaesthetized for as long as it reasonably takes to collect sufficient data to usefully inform the next phase of study. On study completion, the anaesthetized animals will be killed using schedule 1 methods unless the study requires an alternative (previously approved) method.

What are the expected impacts and/or adverse effects for the animals during your project?

In a small number of cases, animals may initially be treated with a drug that could improve

the outcome of the study. The same animals may or may not then have blood samples taken. Then they will be anaesthetised. All the animals will be anaesthetised only once and will be killed under anaesthesia without being allowed to recover. They will not experience anything more unpleasant than treatment with a drug – which is not expected to have any adverse effect – followed by a single injection, usually into their muscles, to sedate them in preparation for general anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals on the license will be anesthetized without recovery.

Studies on the few animals receiving pre-study treatments (protocol 1) and, or blood samples taken (Protocol 1 and 5) are categorized as mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The studies to be described cannot wholly be conducted on isolated organs or tissue, or in anything other than the whole animal.
Which non-animal alternatives did you consider for use in this project?

Elements of most studies will have undergone some in-silico and in-vitro development before progressing to the in-vivo work proposed in this PPL

Why were they not suitable?

The range of procedures conducted under this license are not possible at in vitro levels: whole bodies, or at least, whole organs, either in or ex vivo or ex situ are required. Large animal studies will continue until in vitro techniques, or the use of small laboratory animal species offer greater prospects of study success.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 based on past experience, and increasingly, by reference to the following works :

Moore CG, et al., Recommendations for planning pilot studies in clinical and translational research. Clin Transl Sci. 2011 Oct;4(5):332-7. doi: 10.1111/j.1752-8062.2011.00347.x. PMID: 22029804;
PMCID: PMC3203750

and

Reynolds PS. When power calculations won't do: Fermi approximation of animal numbers. Lab Anim (NY). 2019 Sep;48(9):249-253. doi: 10.1038/s41684-019-0370-2. PMID: 31435053

Typically, 2-10 animals per group will be studied. The number of groups will depend on study design & the hypothesis tested.

Studies will usually be staged, with the least injurious steps being taken first. Cadaver studies will be conducted before some experiments.

The need for on-going experimental refinement AND animal use will be assessed after every study session. This will maximize the rate of improvement of experimental design and protocols. It will also ensure studies are stopped if it is determined unlikely to be successful in the long-term. Imaging and repeated tissue sampling will reduce the variability between animals and help reduce animal use.
Where necessary, statisticians will be involved in the animal–by-animal analysis of experimental progress.

These measures will ensure that unwarranted animal use is minimized

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Study power will rarely be calculated a priori because studies will typically involve small numbers of animal involved in establishing "proofs-of-concept", refining new experimental techniques, and, or improving current models. That said, it is possible that studies on the current application will inform power calculations for subsequent, more focused, larger scale 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 all studies, the model’s value will be continually assessed through an animal-by-animal analysis of experimental progress, and if - after enough animals have been studied - such progress is likely to prove inadequate (as determined by predictive statistics), then the experiment will be suspended, thus reducing animal use. The provision of meticulous anaesthetic care will confine the most important physiological variables within narrow ranges, thus limiting ‘noise’ in experimental objectives. All experiments will be incorporated into an on-going study of the source of variation in physiological variables. Statisticians will be involved when suitably designed studies involve sufficient animal

numbers for analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs are the "default" species because their size, and that of their eyes, lungs, heart, gastro-intestinal tract, kidneys and liver are similar to the human. There is an extensive literature on the use of pigs in experimental surgery and equipment development. The equipment used for humans can be used in pigs without modifications, but not in small laboratory animal species.

Small ruminants will be used when their species characteristics offer distinct advantages over pigs, e.g. surgical access to the calvarium, model precedence, naturally-occurring pathologies.
All anaesthetics will be supervised by specialist veterinary anaesthetists.

All studies conducted under the current application will involve terminal anaesthesia.

Why can’t you use animals that are less sentient?

Pigs and small ruminants are established models for the study of human conditions because their size, and that of their organs, are similar to those of humans. This will offer bi-directional advantages, i.e., equipment currently used in people can be used in the animal model; the animal model can be used to develop new, size-scaled technologies for application to human beings.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Using terminally anaesthetized large animal models in an environment in which experimental refinement is prioritized and a "culture-of-care" constantly promoted will inform the wider research community of refinement techniques in large animal studies.

During each study, the methods used will be assessed on an animal-by–animal basis and improved if opportunities for further refinement are identified. At the conclusion of each study, details of these improvements will be published to assistother researchers.

All animals will be anaesthetized for all studies, from which they will not be allowed to recover. Consequently, they will typically experience nothing more unpleasant than a single injection (two in minority of studies) that in some pigs may cause mild and momentary discomfort. Small ruminants will be expertly restrained for a single, or two sequential intravenous injections.

Anaesthesia monitoring will be refined in most cases by use of the bispectral index (BIS) in addition to other pertinent clinical signs. Analgesia will always be pre-emptive and polymodal.

All anaesthetics will be supervised by an A(SP)A-licensed veterinary surgeon with a post-graduate qualification in veterinary anaesthesia. Anaesthetics may occasionally be managed (under supervision) by A(SP)A-licensed technicians.

During each study, methodological suitability will be assessed on an animal-by-animal basis and refined while recognizing the confines imposed by the need to limit variation. At the conclusion of each study, details of perceived refinements will be published as a matter of laboratory policy.

All studies conducted under the current application will involve terminal anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All studies conducted under the applicant's license will have satisfied the requirements of the PREPARE guidelines (of which the applicant is a co-author). The applicant will provide the corresponding authors of material generated under the PPL with the information they require to meet the ARRIVE reporting guidelines, or, ensure published material is ARRIVE compliant by editing manuscripts himself.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant is an active member of a number of ethics committees/interest groups and laboratory animal associations including 1) The local AWERB; 2) EthicsFirst, an online forum promoting animal ethics; 3) The Animal Welfare, Science, Ethics and the Law Veterinary Association; 4) The Laboratory Animal Veterinary Association; 5) The Laboratory Animal Science Association.

I subscribe to the online newsletters of: 1) the NC3Rs; 2) The Nuffield Council of Bioethics; 3) Understanding Animal Research

152. Regulatory Testing Using Embryonating Hen's Eggs

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- 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

Disinfectant, Efficacy, Extraneous, Agents

Animal types	Life stages
Embryonated Chicken 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 perform quality control batch release tests on poultry vaccines for the absence of extraneous agents to meet the requirements of marketing authorisations under the European Pharmacopoeia 04/2015:20625: Avian Live Virus Vaccines: Tests for Extraneous Agents in Batches of Finished Product 1. Test for Extraneous Agents using Embryonated Hen's Eggs.

Determination of efficacy (or lack of) of disinfectants for approval under the Diseases of Poultry Order 2003 and the Avian Influenza and Influenza of Avian Origin in Mammals (England) Order 2006.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work carried out under this licence helps to ensure the consistency of poultry vaccines, the quality of disinfectants and aids the improvement of 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.

What outputs do you think you will see at the end of this project?

Certificates of Analysis will be produced, based on the previous licence this will be for more than 70 batches of vaccines tested for extraneous agents prior to release and over 100 disinfectant products tested for efficacy.

Who or what will benefit from these outputs, and how?

Batch release testing of vaccines allows pharmaceutical companies to meet their Good Manufacturing Practice requirements to sell their products in compliance with the manufacturing requirements.
Ultimately the work therefore ensures products released onto the market are free from contaminating micro-organisms, thus avoiding the potentially catastrophic causation of a disease outbreak in commercial poultry facilities. Similarly the results of disinfectant efficacy tests provide evidence for manufacturers to submit to competent authorities for authorisation to show their use will be effective in the event of a notifiable disease outbreak.

How will you look to maximise the outputs of this work?

This work is related to production of individual batches of a coccidia vaccine which has a limited shelf life and it is important that the testing is efficient to get it to the market and avoid losses.

The disinfectant work is done for specific compounds for manufacturers and is subject to intellectual property.

Species and numbers of animals expected to be used

- 30,000 (Protocol 1: 7,500 & Protocol 2: 22,500)

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.

Protocol 1. The numbers and ages of the eggs use is specified in the European

Pharmacopoeia, any deviation from these specifications may lead to rejection of the results by the regulatory authorities.

Protocol 2. Although the number of eggs required for this test are not specified in the regulations, the need to accurately determine the titre of any surviving virus means that seven eggs per dilution are considered to be minimum.

Typically, what will be done to an animal used in your project?

Protocol 1. Fifteen eggs (10 test and 5 controls) are each inoculated by 1 of 3 routes. The routes are chorioallantoic sac (CAS), chorioallantoic membrane (CAM) and yolk sac (YS). For CAS and CAM the eggs are required to be 9 - 11 days old at the time of inoculation and for YS they must be inoculated at 5
- 6 days old. The CAS and CAM eggs will be incubated for 7 days and the YS eggs for 12 days. The eggs will be candled daily and any eggs that are unlikely to survive overnight will be euthanised by a Schedule 1 method. At the end of the incubation period all the surviving eggs will be euthanised by a Schedule 1 method.

Protocol 2. Seven eggs will be inoculated for each dilution indicated below by CAS route and incubated for 7 days. The disinfectant can be tested at 1 (as below) or 3 dilutions. The Formalin / Newcastle Disease Virus (NDV) inoculated eggs help assess the validity of the test and the Back Titration confirms the titre of the NDV used. The toxicity test confirms that the disinfectant is not toxic to the eggs.

Disinfectant/NDV: Neat, 10-1 and 10-2

Formalin/NDV: 10-2, 10-3, 10-4, 10-5 and 10-6

NDV Back Titration: 10-4, 10-5, 10-6, 10-7 and 10-8

Toxicity Test. Ten eggs will be inoculated with neat disinfectant and ten eggs inoculated with WHO hard water (Negative controls) by CAS route. (very rarely see any dead eggs, 1 every few months at most)

The eggs will be candled daily and any eggs that will not survive overnight will be euthanised by a Schedule 1 method. At the end of the incubation period all the surviving eggs will be euthanised by a Schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Protocols 1 and 2. Some embryo deaths due to trauma from the inoculation procedure will occur within 24 hours of inoculation but these are below 14 days of age, the age at which the embryo is considered sentient by ASPA.

Protocol 2: Embryo deaths due to NDV will occur mainly between 24 to 48 hours, the majority (~99%) of the remaining embryo deaths will occur before the eggs are 14 days old, the age at which the embryo is considered sentient by ASPA.
Expected severity categories and the 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 work is undertaken in eggs under the age of sentience (or less than 2/3 of incubation period so not a protected species under ASPA) so most will be sub-threshold. Any deaths after 2/3 incubation are classified as mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The procedures detailed in this licence application are governed by European and English regulatory requirements which stipulate the methods of testing to be employed.

The batches of products to be tested under Protocol 1 have the extraneous agents test in eggs listed as a requirement of their marketing authorisations under the European Pharmacopoeia 04/2015:20625: Avian Live Virus Vaccines: Tests for Extraneous Agents in Batches of Finished Product. 1. Test for Extraneous Agents using Embryonated Hen's Eggs.

Embryonated eggs have historically been seen and are still considered to be the most sensitive method for the culture of NDV and are still in routine use for diagnostic purposes. They have also historically been the system of use for the Defra disinfectant test.

Which non-animal alternatives did you consider for use in this project?

Protocol 1. Eggs must be used.

The vaccines have the extraneous agents tested in eggs listed as a requirement of their marketing authorisations and the European Pharmacopoeia.

Protocol 2. Cell cultures have been considered.

Eggs have historically been seen and are still considered to be the most sensitive method for the culture of NDV and are still in routine use for diagnostic purposes.

Why were they not suitable?

Cell cultures have been trialled but there were issues with toxic effects of disinfectants on the cell cultures.

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of eggs is based on the expected number of batches of vaccine for Protocol 1 and the number of disinfectant samples for Protocol 2.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Protocol 1. The number of eggs used for each test is the minimum number required by the European Pharmacopoeia. Any deviation from these numbers may lead to rejection of the results by the regulators.

Protocol 2. Although the numbers of eggs for this test is not specified in the regulations, the need to accurately determine the titre of any surviving virus means that seven eggs per dilution are considered the minimum.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

See above, the number of eggs to be used is dictated by the legislative requirements.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 detailed in this licence application are governed by European and English regulatory requirements which stipulate the methods of testing to be employed, thus limiting the opportunities for refinement to competent and experienced staff in the inoculation and candling of eggs.

Why can’t you use animals that are less sentient?

No alternative available. Embryonated eggs are specified by the regulations and those used below the age of sentience.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Eggs will be candled daily and any that will not survive overnight are euthanised immediately.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

See above.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

N/A

153. Immunity and Cardiovascular Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cardiovascular Disease, Immune System, Inflammation, Nutraceuticals, Therapeutics

Animal types	Life stages
Mice	adult, aged, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

- To improve our understanding of the complex role that the immune system plays in cardiovascular disease.
- To evaluate the anti-atherosclerotic potential of various therapeutics ranging from monoclonal antibodies through to dietary supplements such as probiotics and purified plant extracts.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work covered within the remit of this licence aims to further our understanding of the role that the immune system plays in cardiovascular disease. This is important because despite the advent of statins and other lipid lowering drugs the incidence of atherosclerosis, the main underlying cause of cardiovascular disease, remains very high and cries out for the development of further treatments.

The project covers several areas of immune involvement in cardiovascular disease. These include developing a greater understanding of how immune signaling molecules called cytokines control and direct white blood cells to the sites of atherosclerosis, understanding the mechanisms and consequences of how the part of the immune system known as “complement” interacts with fat metabolism and finally developing and testing possible therapeutic agents to block the adverse effects of immune involvement in atherosclerosis.

What outputs do you think you will see at the end of this project?

Through the course of this program we hope to generate interesting and novel data regarding basic biology of the role of immunity in cardiovascular disease. Employing a dual track approach we shall also test numerous potential therapeutics ranging from monoclonal antibodies targeted at parts of the immune system involved in the disease process, through to plant based nutraceuticals and probiotic formulations all with proven activity in dampening down inflammation and the immune response. The data and information arising from this work will be published in scientific journals and presented at relevant conferences.

Who or what will benefit from these outputs, and how?

We anticipate that these studies will increase our understanding and help clarify the complex role that the immune system plays in the development of cardiovascular disease. Furthermore, through projects testing and analysing a wide range of anti inflammatory agents we shall endeavor to identify and validate new therapeutics that will merit further work and potential testing in humans. The possible benefits are large in that completely new approaches to the treatment of cardiovascular disease may emerge. This is important since despite all the advances of the last few decades the disease remains the extremely common and is a huge burden on healthcare systems globally.

How will you look to maximise the outputs of this work?

We will maximise outputs from this program through collaboration and rapid dissemination of knowledge by publication. We are already collaborating with three different groups on different aspects of cardiovascular disease and we are always interested in working with colleagues to further our understanding of the complexities of cardiovascular disease since it is through such work that often new ideas and new avenues of potential therapies appear.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The model mouse strains that we shall use are very well established (~25years) models of human cardiovascular disease having been used in thousands of studies since their generation in the mid 1990's. We will use these models in combination with various other

strains to investigate the role of genes from the immune system in heart disease.

Typically, what will be done to an animal used in your project?

A typical experiment might proceed as follows: genetically modified model mice would be randomly assigned to experimental and control groups and placed on a high fat diet at 8-10 weeks of age. In some cases these animals might have small blood samples taken to allow us to determine baseline levels of cholesterol and immune markers. Experimental groups could have their diet further supplemented with a nutraceutical such as catechin from green tea or a probiotic formulation. After 12 weeks all animals would be humanely killed and tissues taken for analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect that the vast majority of our mice (>90%) which have been fed a high fat diet over a period of 12 weeks will experience no adverse effects whatsoever. In fact these animals will fall below at or below the “mild” category.

Expected severity categories and the 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 prolonged periods of high fat feeding (4-6 months) up to 25% of the mice may develop itchy skin causing them to scratch, resulting in sores. These animals we would classify as falling into the “moderate” category of severity. However, we have refined our experimental design to an extent where we only rarely (<10%) use such long periods of high fat feeding currently with our standard experimental timepoint of 12 weeks high fat feeding the incidence of itchy skin and sore has fallen to 1.3%.

Approximately 0.92% of mice placed on a high fat diet for 12 weeks will die suddenly and without any prior warning. These deaths are almost certainly due to heart attacks or strokes and are mirrored by similar occurrences in humans where very often, the first apparent incidence of heart disease is a heart attack or stroke. In the mice, these deaths almost always occur unobserved so we cannot say for certain what level of distress the animal experiences before death. Because of this all these occurrences are graded “severe”. The protocols do not have a prospective severity of severe due to the very low expected incidence of this.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While it is possible to examine the effects of single agents such as individual cytokines on cells in vitro(eg a macrophage cell line), the biological relevance of these results have to be tested in vivo. This is because the complex immune, cellular and biochemical interactions that occur between the different cell types in the context of the artery wall and the atherosclerotic plaque simply cannot be modelled in isolated cells.

Thus, studies in animals are unavoidable if we are to understand the detailed pathology of atherosclerosis and design new therapies to combat this disease which still accounts for almost half of all mortality in the developed world.

Which non-animal alternatives did you consider for use in this project?

Wherever possible we use in vitro studies before considering whether to carry out animal work and these studies have yielded (and will continue to yield) important insights into the effects of complement activation and cytokine stimulation of the different cells types present within the atherosclerotic plaque.

Why were they not suitable?

While very useful, the normal context within which the immune system is activated is missing and hence it is dangerous to extrapolate too far the results gained from these studies to the situation which occurs in humans. Thus the necessity for 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?

I have looked at the last iteration of the licence and given that we only used ~28% of the numbers allowed I have reduced the total numbers needed by 1000 from 3500 to 2500.
What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Two key factors will aid us in reducing the number of animals to be used in the proposed work.

Firstly, rigorous in vitro work and analyses of the literature leading up to the statement of a hypothesis will ensure that the proposed experiments to test each hypothesis are focused and have clear goals.

Secondly, our experimental design and data collection was based upon disease models for which reproducible protocols for quantifying pathological changes have been established involving immuno- histochemical studies of the arteries from the mice involved. In each experiment, group sizes will be selected based on the efficiency of disease induction and utilising statistical advice. The reproducibility that has been achieved using the described protocols is key to minimising the numbers of animals required in the studies. Animal usage is further reduced, whenever possible, by designing sets of

experiments to share control groups. Furthermore, the application of modern imaging technologies (eg PET and MRI) allows repeated data collection from the same animals. This removes the need to have individual groups at each time point and reduces variation within groups allowing us to use smaller group sizes.

We estimate that most of our experimental studies comparing double knockout animals with single knockout controls will require group sizes of 15. We base these assumptions both on power analyses and experimental findings from previous work carried out under three previous project licences. We have found that this is the minimum number of animals required to be able to show that the differences in plaque size between groups are statistically valid.

However, where we wish to examine the effect of specific immune related genes or cells on the incidence of plaque rupture we may require group sizes to be increased to 35-40. We base this on the work of one of our collaborators, who has many years' experience studying plaque rupture in the mouse models of atherosclerosis and has shown that this is the minimum number of animals allowing properly powered statistical analyses (with $p<0.05$ and a power level of 80%) of plaque rupture and the factors affecting it.

Wherever possible we shall use groups of mixed sex to reduce wastage related to breeding animals that will not be used experimentally. However, in some cases, where the biology of gender affects a specific study we may need to either choose one sex or both.

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 with genetically modified mice will utilise either existing or newly generated double (or triple) knockout (and control) colonies of mice. These paired colonies are generated from the same breeding program and thus provide ideal test and control groups with virtually identical backgrounds thus reducing animal numbers required.

Wherever possible we shall employ pilot studies with therapeutic agents under test as a stop/go indicator. Those agents showing no or little efficacy shall be abandoned at this stage without further larger scale experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The model mouse strains that we shall use are well established models of human cardiovascular disease. We will use these models in combination with various other knockout strains to investigate the role of genes from the immune system in heart disease. We now have several years experience in using these models and this together with knowledge shared from our collaborators and gleaned from published work has allowed us

to refine our use such that we expose mice to high fat diet for considerably less time than the licenced period of 12 months. Indeed the vast majority of our experiments are limited to 12 weeks duration. This refinement keeps both the incidence of skin sores (1.33%) well below the expected 25% present after 4-6 months of high fat diet and also restricts the incidence of sudden cardiac death to 0.92%. Experimental animals are monitored closely to ensure that any which show any signs of cardiovascular problems are humanely and rapidly euthanized.

Why can’t you use animals that are less sentient?

The development of heart disease is a very complex process involving a host of different systems and processes within the human body. Some of these can be studied in vitro in the laboratory and within the human population. However while these approaches can provide many extremely important insights and are critical in informing all work carried out with animals, they remain largely incapable of mimicking the interactions which occur during the evolution of the disease within the body. Thus it remains essential to use rodents in the study of heart disease. While is possible to use less sentient species for some specific aspects of cardiovascular research it is currently impossible to model the complexities of the mammalian immune system and its impact on the development and progression of atherosclerosis in lower life forms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Through our careful approach to our work we have already made significant improvement sin refinement over the course of the last PPL. Namely through a focus on shorter duration experiments, more reliance on the less aggressive LDLr model we have reduced the incidence of sudden cardiac death in our mice from 4-7% in previous licences to 0.92% in the last iteration. Alongside this success the number of animals suffering from itchy skin and resulting sores has also fallen to 1.33%. We shall employ the same rigor during the next five years to ensure that these benefits are maintained.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We shall monitor all relevant sources of literature and consult widely within our institution to ensure we maintain best practice and the highest standards of animal welfare and experimental refinement possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We shall appraise ourselves of the literature and information available on the NC3R website as well as making regular effort to search the general literature for relevant advances in the 3Rs. We shall seek to attend local and national events relating the the 3Rs and where feasible devise and seek funding for projects promoting the principles of Reduction, Replacement and Refinement.

154. The study of inflammation, thrombosis and immunity in relation to atherosclerosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - (ii) 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

atherosclerosis, cardiovascular disease, inflammation, immune system, thrombosis

Animal types	Life stages
Mice	juvenile, adult, 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 gain an increased understanding of the mechanisms of vascular inflammation and thrombosis (blood clotting) in relation to atherosclerosis, as well as the role of the immune system and accompanied potential imaging and therapeutic targets.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular disease, mainly comprising heart attacks and strokes, is the leading killer worldwide. According to the latest figures there are currently around 550 million people living with heart and circulatory diseases across the world. In the UK, one in eight men and one in thirteen women die from coronary heart disease. Coronary heart disease is caused

by many factors, this includes inherited ones as well as acquired by smoking and/or a poor diet. However a key component of the cause of coronary heart disease is the involvement of the immune system and its interaction with the cardiovascular system.

The common underlying cause for cardiovascular disease is atherosclerosis, which is the build up of fatty substances in the walls of blood vessels. These fatty substances, or plaques, can accumulate in the vessel walls to such an extent that they can limit blood flow, restricting blood flow to vital organs. As well as causing blood vessel narrowing, these atherosclerotic plaques may rupture, or break open, exposing their fatty core and leading to the formation of a blood clot. This blood clot, when combined with the ruptured plaque, can cause complete blockage of blood flow down blood vessels. When this occurs in the coronary or carotid arteries (the blood vessels that supply the heart or brain, respectively), this can potentially result in a heart attack or stroke. It is of vital importance to investigate the multiple factors that lead to atherosclerosis and related biological processes which lead to heart attacks and strokes. There is an urgent need for diagnostics, predictive tools, treatments as well as new targets to be investigated in order to translate interventions to humans. Any increased understanding or new treatment approaches will have massive impacts worldwide, given the huge burden of cardiovascular disease.

Inflammation plays a major role in all phases of atherosclerosis. Plaques can be refered to as stable or vulnerable. Stable plaques can occur through the build up of fatty deposits in the walls of the coronary arteries, which carry oxygen and nutrients to your heart, causing them to become thickened and stiff.
Vulnerable plaques can rupture and lift off of the coronary artery wall leading to a clot, which then causes a heart attack or stroke. Stable plaques are characterised by an inflitration of inflammatory cells, whereas vulnerable plaques are caused by an active inflammation, which involves thinning the fibrous cap, which is the structural support of the plaque, causing rupture to occur. These inflammatory cells are also involved in immune system functions and therefore understanding how these aspects interlink can help our research progress.

We aim to explore cutting edge science to aid in the diagnosis and and treatment of dangerous plaques that are responsible for the majority of heart attacks. We believe that our work will have a significant impact on human health in the foreseeable future.

What outputs do you think you will see at the end of this project?

Our research will provide data that will be novel scientifically and furthermore will be used for the development of new diagnostic and therapeutic targets in preventing and treating the causes of heart attacks and strokes.

New information will be published in peer-reviewed scientific journals and will inform scientists and clinicians working on the development of improved techniques for the diagnosis and treatment of patients with cardiovascular disease. In addition to publication the data will be presented to the scientific community in 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 the basic pathology and physiology that leads to arterial heart and vascular disease.

In the medium term the project aims to develop new diagnostic and therapeutic targets that

will be useful for future research and other investigators conducting experiments in a similar area. If successful and we can identify new diagnostic targets in atherosclerosis then we will use these to try to design new experimental tailored treatments. Furthermore data will be used to design experiments and investigations that will move the field forwards in future work.

In the long term, doctors will benefit from the development of improved techniques for the diagnosis and treatment of patients with cardiovascular disease. Furthermore, patients may directly benefit through a reduction in the number of heart attacks and strokes and hopefully deaths.

How will you look to maximise the outputs of this work?

We will communicate our work via scientific publications, public engagement as well as via targeted seminars and national and international research meetings. We will communicate via our establishment communications team to ensure adequate dissemination of our work both to local and international stakeholders.

Species and numbers of animals expected to be used

- Mice: 3400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

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 that develop fatty substances or plaques in the blood vessel walls at an accelerated rate. In order for this to happen, the mice are genetically altered so that they lack an important fat handling receptor. This allows us to produce a good atherosclerotic model of the human disease, of different stages, by giving them a high fat diet. Performing studies at different ages with different durations of high fat diet and different interventions will allow us to study the pathology thoroughly and in a way that reflects the human disease over the years.

The use of this model 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 fed a high fat diet for periods of up to 14 months. Some experiments will involve comparing aged matched animals on high fat diet vs non high fat diet undergoing different interventions. We will study progression and regression of disease using this model.

Animals may be injected in order to induce inflammation or be administered substances

such as drugs, proteins, antibodies, nanoparticles, genes or other agents in order to modify specific outcomes.

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), single photon emission computed tomography (SPECT), computed tomography (CT), magnetic resonance imaging (MRI) or fluorescence molecular tomography (FMT).

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.

Potential adverse effects include: skin damage from high fat diet, infection from administration of treatments, blood loss from blood sampling and the reddening of skin from prolonged exposure to various substances.

As a consequence of the high fat diet and the genetic strain of mice that we will be using, the mice may experience heart attacks or strokes that may occur spontaneously. However we will carefully monitor this and where possible reduce this risk.

Expected severity categories and the 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 or moderate severity categories.

We expect 75% of our mice to fall under the mild category and 25% of our mice to fall under the moderate category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The use of animals is essential for our work, since it is only by study of a live animal that we will be able to work out how atherosclerosis is caused, how it can be most effectively and accurately diagnosed, and what therapeutic approaches might be of benefit.

Which non-animal alternatives did you consider for use in this project?

We considered and will use cell culture experiments (as well as other scientific techniques) as much as possible. These will include using cells derived from genetically-modified mice bred in the project.

We also have considered doing human studies.

Why were they not suitable?

Cell culture experiments are suitable models. However, experiments in a whole animal are also necessary, as laboratory techniques cannot mimic the complexity of the whole animal, nor replicate human clinical scenarios as closely. Nevertheless, we will make maximal use of preliminary laboratory experiments to refine questions and protocols prior to conducting specific animal experiments.

We also have considered doing human studies, however for our aims this would be impossible as we are exploring early technologies that are not yet licensed for human use and therefore animal experiments are required.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated mice based on the anticipated numbers of experiments, the numbers of experimental groups and the numbers of mice in each group. With the considerable variability of our previous experiments taken into account and within reference to the published relevant studies, we have accounted for a sample size of between 6-12 animals in each group to be studied for each experiment planned. This is based on calculations which are also included within the grant applications relevant to this license. Therefore, we have concluded that we need to use 800 mice for protocol 2 and 100 mice for protocol 3. In terms of the breeding numbers of 2500 (protocol 1) this is based on experience and having taken advice from the animal team.

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 which we have developed are generally extremely reliable and reproducible, so we can obtain scientifically significant results from small groups of animals.

Animal numbers may also be reduced by the application of whole body imaging techniques that allow study of animals on multiple occasions and thereby avoid killing different groups of mice at each time- point of an experiment. In addition mice will be used for imaging whilst they are alive, as well as a detailed assessment of all tissues after

death. This ensures that we get as much data as possible from each mouse and thereby limits the number of mice needed.

Furthermore, we will consult 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 amount of animals and that each animal is being utlised to it's full potential.

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 performed, prior to a larger study, for every new diagnostic and therapeutic atherosclerosis target. These can reveal deficiences in the design of an experiment or procedure which can then be addresssed before animals are expanded on a larger scale study.

The number of animals used can be optimised through a multiple time point study, which details progression of atherosclerosis over a period of time, using the same group of animals.

We will monitor our breeding colony to ensure that we are correlating our sample sizes for experiments with the amount of animals being produced, to avoid breeding unnecessary 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.
Animal models

Mice are the most suitable for our project for a number of reasons which are:

They have a well researched and understood immune system which is an advantage for our study on the role of different diagnostic or therapeutic targets.

There is a wide variety of mice that are readily avaliable as genetically modified. Genetic modification is a technique that changes the characteristics of an animal by removing or adding specific genes. This can help us determine the effect of specific targets within the blood vessels and atherosclerosis. There has been in particular a major advance in treating coronary heart disease using the animal models that we are proposing in this license and we hope that with these validated models we can make further significant advances.

There is a variety of modern imaging equipment that is avaliable for use in small animals such as mice, which can allow us to evaulate our molecular targets in action.

Methods

Animals will be fed a high fat diet so that they can develop sufficient amounts of atherosclerosis. This is the most natural way of inducing this disease and resembles the human model the most.

To administer injections we will ensure that the mice suffer as little as possible. For example, we will ensure that the restrainer is cleaned prior to avoid inducing extra stress in the mice caused by prior experiments. The equipment used to restrain the mice are designed well to hold mice comfortably and are red in colour, in order to reduce stress as mice cannot perceive this colour. As injection can be uncomfortable, we will use an anaesthetic cream (EMLA 5%) which can numb the area in order to reduce the irritability of the injection. Tail vein dilation is also stimulated using warm water or a warming chamber to increase the likelihood of getting the injection correctly placed first time to reduce the number of attempts; thus reducing the stress for the animal. Similar methods are used whilst blood sampling. In addition whilst blood sampling the smallest amount of blood will be collected and none wasted. We consider the NC3Rs blood sample in mice decision tree to ensure we are using the most appropriate blood sampling technique found.

Substances that will be injected to either accelerate or reduce the disease process would have been tried previously in different animals/humans and we will do a thorough literature review prior to use. Other means of introducing different substances to the mice might be used, such as by mouth or by surgical implantation of a device that releases a substance over a prolonged period of time.

Hair removal cream is not left on longer than 2 minutes in order to reduce potential skin reddening and shavers are used prior to this to ensure the maximum amount of hair is removed without pain. This procedure is done whilst the animal is asleep under general anaesthesia.

General anaesthesia to put the animal asleep, is commonly used within our protocols. To ensure that the animal is without pain we regularly check for the effectiveness using the toe pinch reflex. If a reflex is found the amount of anaesthesia is increased and then regularly assessed. This is of upmost importance whilst animals are being scanned and we check their heart rate and pain levels whilst scanning.

Why can’t you use animals that are less sentient?

Mouse models of atherosclerosis are common and well validated. There are no high fidelity animal models for atherosclerosis 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 atherosclerosis in high fidelity models.

Non-protected animals (e.g. invertebrates such as insects, decapods, nematodes) cannot be used to meet our objectives as they do not replicate a human model of atherosclerosis as closely as a mouse. Less sentient animals (eg zebrafish) are suitable for investigating molecular mechanisms, however they would not be suitable for studying clinically relevant atherosclerotic lesions, whereas the mouse model would be the least sentient model where clinically relevant studies can be undertaken.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for the animals?

Our procedures are all reviewed ourselves and by NACWO and NVS to ensure we are minimising animal welfare costs.

As our atherosclerosis mouse model with high fat diet can cause spontaneous heart attacks and strokes we have increased the monitoring of our animals by our team. Our team and the technicians that closely work alongside the animals are also aware of this and therefore the animals are throughly checked for signs of ill-health. In addition the animals are also carefuly monitored for signs of pain or distress and poor welfare.

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. In addition the mice are warmed during induction of anaesthesia and during hair removal through use of a heating pad. The hair removal apparatus is also warmed prior to reduce temperature changes in the animal.

A recent review we conducted showed that by placing high fat diet on the floor of cages, instead of in the hoppers, this minimised the chances of sores on the skin of animals. This review involved increased monitoring of animal by technicians and ourselves to ensure that the refinement was working. We are still researching whether this can be even more refined through different housing apparatus.
We recently reviewed pain management in our animals whilst administrating substances and through suggestion of the named training and competency officer, local anaesthetic (EMLA 5%) cream was purchased to numb the area prior to injection.

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?

We will follow the latest guidance available from LASA, PREPARE and the latest ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly view the NC3Rs website 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.

155. Mechanisms for immunological memory

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Immunisation, Cancer, Autoimmune disease, Nematode, T-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 project will aim to investigate mechanisms of immunological memory that are induced upon immunisation and when fighting against infection and cancer, while avoiding overreactions to self- tissues (i.e. autoimmunity). Particularly, the project will focus on a special type of lymphocytes(white blood cells), named T-cells, which can specifically recognise antigens and retain immunological memory.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

T-cells are required to produce specific immune responses to antigen (immune-reactive molecule of pathogen or non-pathogen including self-tissue). Activities of T-cells are essential for fighting infection and cancer, in which T-cells are often suppressed by their own negative regulatory mechanisms. On the other hand, overreaction of T-cells can induce inappropriate immune responses, which lead to disease conditions such as allergy and autoimmune disease. In addition, hyperactivities of T-cells can worsen infection,

leading to a stronger inflammation and a prolonged disease (e.g. severe COVID-19). Thus, it is important to understand how T-cells control immune responses, particularly, why T-cells can become unresponsive in those disease conditions, while T-cells can become overreactive and unfocused in some other disease conditions. Notably, these apparently different diseases, including infection, cancer, allergy, and autoimmune diseases, have shared T-cell-mediated mechanisms. Therefore, the understanding of mechanisms underlying T-cell activities will improve strategies to treatment and prevention of these diseases, including vaccination for infection and cancer.

What outputs do you think you will see at the end of this project?

Improved understanding of how T-cells react to antigens and thereby induce effective responses to protect the body, and how they induce autoimmune reactions. Specifically, we will reveal how the actions of individual antigen-reactive T-cells change over time.

We will have 5 major publications (at least one publication from each objective).

We will identify new mechanisms of memory T-cell responses which can be further investigated for future clinical application to vaccination and diseases including autoimmunity, allergy, infection and cancer.

Who or what will benefit from these outputs, and how?

Short-term beneficiaries include the following:

Academic researchers in immunology, infectionology, parasitology, cancer biology, allergology, and related clinical disciplines including rheumatology, respiratory medicine, and dermatology.

Industrial researchers in pharmaceuticals and biotechnology.

Long-term beneficiaries include clinical practitioners, patients, pharmaceuticals, and biotech companies.

How will you look to maximise the outputs of this work?

Collaboration: the outputs of the proposed work will be maximised through my active collaborations in parasitology, cancer immunology, haematology and virology.

Clinical translation: I will have regular meetings with my clinical collaborators.
Dissemination: I will liaise with Communications offices of my institute and funders.
Publication of unsuccessful approaches: I will aim to publish short reports in appropriate journals

Species and numbers of animals expected to be used

- Mice: 4600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most appropriate species, because they are the most established model for investigating immunological memory and tolerance, and have all relevant subtypes of lymphocytes for humans, and have rich scientific resource such as antibodies. In addition, because GM animals are required for this project to investigate the gene functions, and mice have the broadest repertoire of GM available.

The immune system shows dynamic changes throughout the juvenile and adult stages, which correspond to child, adult and aged people. Thus, mice in these life stages will be experimented using a disease or immunisation model.

Breeding protocols will use all the stages of mice apart from aged mice.

Typically, what will be done to an animal used in your project?

The majority of the experiments in the project will be carried out using mice as a source of tissue only. We propose to do mild procedures only, wherever possible. The standard breeding protocol (Protocol
1) will cover:

Breeding of mice that may show a harmful phenotype due to immune dysregulation and autoimmune inflammation. Affected mice start to show clinical symptoms around 10 days after birth.

Some animals will receive procedures for one of the following models.

Immunisation model: typically mice will receive two subcutaneous (under the skin) injections and be humanely killed and analysed within 4 weeks after the start of experiment.

Skin allergy model (Contact Hypersensitivity): typically mice will have a skin allergen painted on their skin twice (i.e. sensitisation and challenge) and be humanely killed and analysed within 2 weeks after the start of experiment.

Multiple Sclerosis model (Experimental Autoimmune Encephalomyelitis, EAE): experiments will analyse the disease onset and thereby minimise welfare costs to animals. Animals will be humanely killed once the disease onset is confirmed (i.e. this will be either mild or moderate severity), and their organs and lymphocytes will be analysed. All these animals will be humanely killed and their tissues will be analysed within 1 month after the start of experiment.

Cancer model: some animals will be inoculated with a tumour cell line by subcutaneous injection and their immune response to cancer will be analysed. These animals will be humanely killed and their tissues will be analysed before tumours grow to a relatively small size, and thus, the typical duration is less than one month.

Asthma model (Airway Hypersensitivity): typically mice will receive an antigen by subcutaneous injection and thereafter an intranasal administration of the same antigen (i.e. challenge) and be humanely killed and analysed 3 days after the challenge.

Parasite model: some mice will be infected with a natural parasite (precisely, Heligmosomoides polygyrus) through the oral route. Some of these mice will receive second infection typically 4 weeks after the first infection and be observed for up to another 2 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Immunisation model: typically mice do not show any obvious change. Rarely, mice will develop skin ulcer, in which case they will be promptly humanely killed.

Contact Hypersensitivity: mice may develop a mild swelling and redness of the skin, which last until the end of experiment (i.e. within 2 weeks after the start of experiment).

EAE: typically mice develop a mild weakness of the tail or a hindlimb, and some mice may lose a balance or show a reduced weight, and they will be promptly humanely killed and analysed once they develop any symptoms that constitute a moderate severity. Mice typically develop a symptom 2 weeks after the start of experiment and therefore will not experience any effects more than 2 weeks, since all experiments will be terminated 4 weeks after the start of experiment.

Tumour: mice will show a small lump at the site of injection. Typically mice do not develop any other symptom and will be kept until the tumour reaches a certain size as shown in the protocol. The maximum duration is dependent on which tumour cell line is used, and is 4 weeks on average. Rarely mice show a reduced weight or skin ulcer, in which case they will be promptly humanely killed.

Parasite: typically mice do not show any symptom. Rarely mice will develop diarrhoea and a reduced weight, when mice will be humanely killed promptly.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: Moderate (17%), mild (34%), subthreshold (49%)
What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project will investigate mechanisms of a special type of lymphocytes, T-cells. Each T-cell has a unique T-cell receptor, which is an 'eye' to recognise its target (precisely, antigen) and is generated by random recombination of the T-cell receptor gene in the thymus. Thus,

any antigen can be recognised by some of T-cells in the body, and only a small number of T-cells react to each antigen. This unique regulation is fundamental for memory T-cell responses, and cannot be analysed without the use of animals.

Which non-animal alternatives did you consider for use in this project?

In vitro assays, in silico (i.e. using computer) methods, analysis of blood from human patients.

Why were they not suitable?

Currently no in vitro assays can recapture the diversity of T-cell responses, and no in silico methods are available for predicting T-cell responses.

Results that can be obtained by human patients are 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?

The project aims to investigate mechanisms of immunological memory and address the five Objectives. It will be required to ~400 animals for each Objective, since typically 4-8 questions will be asked in each Objective and each conclusion can be drawn by experiments using 50-100 animals. Thus it is estimated that we will use 2100 animals for experiments.

Since we use GM mice which were generated in our past projects, most animals will be obtained by breeding animals in the animal facility of our institute using the breeding protocols in this project license. Some GM mice are maintained as heterozygotes and thus we will genotype mice and use mice with appropriate genotypes for experiments. It is estimated that 2500 animals will be used for generating mice to be used for experiments while maintaining our mouse colonies stud and obtaining appropriate genotypes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design and power calculations were made using 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?

Pilot experiments. This allows us to carry out informative experiments only.

Computational and pharmacological modelling, wherever possible, to determine and minimise the time points to be analysed. GM animals will be used, and this will allow more

efficient and indispensable ways to achieve the 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.

Immunisation and skin allergy models will be used because these models provide valuable data for T- cell responses while minimising suffering of animals.

A neuroinflammation model will be used in a restricted and modest manner in order to investigate T-cell responses during neuroinflammation in the central nervous system specifically at the onset of disease. Thus, the suffering of animals will be minimised by terminating experiments just at the time when T- cells start to infiltrate the spinal cord.

A tumour inoculation model in the subcutaneous space will be used, because this is the simplest and shortest method for investigating T-cell responses to cancer.

The nematode Heligmosomoides polygyrus (H. polygyrus) will be used as a model of parasite infection, because H. polygyrus is a natural and mild parasite for mice and causes minimal suffering, without leaving any recognisable symptom in most of animals.

Airway inflammation model will be used using a protocol with a relatively short duration, so that animal suffering will be minimised.

Why can’t you use animals that are less sentient?

Mice have all human-equivalent immune cell subsets and share most major mechanisms of the immune system, including T-cells, with humans. Experimental resources are extremely well developed for investigating mouse immune cells including T-cells. For example, all major immune checkpoint inhibitors were developed using mice and currently widely used for treating cancer patients. On the other hand, non-protected animals (i.e. invertebrates such as insects, decapods, and nematodes) do not have T-cells and thus cannot be used for addressing any objectives in the proposed project.

The T-cell system starts to be developed in the peripheral immune system after birth and is matured around 3- 4 weeks after birth. Mouse T-cells show equivalent ageing to human adults' T-cells when mice become 6 weeks old - 3 months old.

The majority of T-cells are in the resting state, while antigen-specific T-cells initiate their first responses only after antigens are administered to mice. In addition, such antigen-experienced T-cells become memory T-cells several weeks after the initial exposure and make memory T-cell responses upon encountering with the same antigen. This project investigates such T-cell responses using foreign antigen (immunisation) cancer, self-antigens (myelin protein), and infection. Thus, it is required to experiment on animals using

these disease and immunisation models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will increase the frequency of monitoring as appropriate, seeking advice from vet, animal care technicians. For example, when we expect that mice may develop a disease with a significant symptom(s) (e.g. tumour, neuroinflammation), we will monitor mice typically once a day by observing their gross appearance and behaviours, checking expected clinical signs, and weighing them to analyse their body weight change.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow appropriate NC3R's publications and the latest LASA guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To be informed through circulation emails from the 3R communications office of our institute, and attend any relevant seminar or course if required.

156. Role of chromatin regulators and transposon control in early development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

stem cells, chromatin, transposable elements, epigenetics

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 further our knowledge of how cells regulate the packaging of their DNA during development, including how they control specific parts of the DNA known to be mobile, called transposable elements.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 early development, a single fertilised egg proceeds through a series of steps to develop into a multicellular embryo, called a blastocyst, which contains the three main types of cells - germ layers - to eventually make up the entire organism. These earliest steps require a significant degree of repackaging of the embryo's genetic code, DNA, which is wound around proteins called histones and stored in the cell as chromatin. In essence, this chromatin remodelling changes how easy it is to "read" different parts of the embryo's genetic code and is essential to make sure that from one single cell the hundreds of specialised cells within an adult organism are eventually made. How this remodelling occurs in the early embryo mechanistically is still poorly understood. Characterising the mechanisms that regulate early development is essential to determine how healthy embryo development proceeds, and to shed light on might go wrong in disease or infertility.

Our work focuses on uncovering the role that chromatin and transposable element regulation plays in normal development, and also how such regulation fails in pathological processes such as aging/senescence or cancer. This is important because we still do not understand the mechanisms that regulate these processes. In particular, we are interested in transposable elements – DNA sequences able to copy-and-paste themselves into new places in our genome – because they have only recently been implicated in several developmental and disease processes and are thus significantly understudied. Our previous work in mouse embryos and in cells derived from embryonic blastocysts, called embryonic stem cells (ESCs) has highlighted that there is much more to be understood about the mechanisms that regulate transposable elements and chromatin organisation in healthy cells.

What outputs do you think you will see at the end of this project?

We will generate important information about the role of chromatin regulators and the control of transposable elements in early embryogenesis. This will be shared in the form of data sets and peer- reviewed publications, as well as conference presentations before and after publication. We will also generate and/or characterise several new mouse models of specific factors, which will be available in future for collaborative work and further study.

Who or what will benefit from these outputs, and how?

Our work will lead to an increased understanding of early embryogenesis and specifically, how chromatin regulation is important for gene and transposon regulation during development. These data will be published and thus then benefit the scientific community, who will have access to our findings that may then inform their own research. Prior to

publication, results will also be presented at conferences. Together this will enable scientists to request the mouse models we generate, which will be available upon publication of the relevant findings, or on a collaborative basis before publication. They will then be able to study the function of these proteins within their own biological questions that are outside the scope of our research, maximising the benefit of the mouse models for the wider scientific community. Long-term, since transposon mis-regulation is associated with pathologies such as cancer, neurodegeneration, inflammation and senescence, insights gained in our work may also inform the study of disease, and could lead to new therapeutics.

How will you look to maximise the outputs of this work?

Prior to publication we will present at national and international conferences to disseminate our research, and aim to deposit manuscripts on a preprint servers. We will publish Open Access wherever possible to maximise the availability of our findings. We will share our mouse models and establish collaborations to study gene function in biological contexts outside the scope of our research. Analysis scripts important for the interpretation of our data will continue to be freely available in our account within public code repositories and on our website. We will continue to share and communicate our research on social media platforms and through outreach programmes. We also have funding through the UK research councils to explain our science to a broader audience in an accessible fashion.

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 the best characterised model system for studying early mammalian development, and many developmental paradigms were uncovered through mouse embryo research. Several aspects of chromatin and transposon regulation in development have been shown to be similarly controlled in mouse and human, meaning that experiments here may also be highly relevant for understanding early human development. Animal research is a valuable and essential part of our work, because the best and lowest species model for early mammalian embryo development is the mouse embryo. While 3D models generated from embryo-derived stem cells (ESCs), called organoids, are showing promise for particular areas of research, there is no satisfactory model for very early embryos. In particular, we study the reprogramming events that occur right after fertilisation of the egg, which cannot be fully modelled in ESCs. Therefore we need to use fertilised eggs from mice, which we can then culture further in the lab. We will breed mice and collect these early-stage embryos for our research.

Typically, what will be done to an animal used in your project?

In most experiments, mice will be bred in timed matings, then females humanely killed for embryo collection. Alternatively, non-pregnant females will be killed and then unfertilised

eggs (oocytes) collected and studied directly or fertilised in a dish, to allow closer control of the timings of fertilisation. Early embryos will be cultured in a dish in the lab until they reach the desired developmental stage and then analysed. Prior to mating and/or killing, females may receive hormone injections to stimulate them to produce more eggs (superovulation), which will overall reduce the number of mice needed to obtain sufficient embryos for study. These procedures will be done on wild-type mice as well as genetically altered animals, allowing us to study the effects of select alterations upon embryo development.
Occasionally, females may be injected with a substance during pregnancy to induce gene deletion at a specific stage of their embryos' development.

Where the required mouse models to study a factor are not already available, we will generate one. For this, genetically altered embryos may be surgically or non-surgically implanted into the uterus of pseudopregnant females (females mated to sterile males). The resultant offspring will be founder animals and will be subsequently bred to generate a mouse line.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals are expected to feel no negative impact apart from mild and temporary discomfort at injection sites if substances are administered (mild).

Animals that undergo surgery for embryo implantation or vasectomy are expected to make a rapid recovery within two hours, with any sign of pain or distress rare.

Where pregnant females are dosed with agents to induce gene deletion, adverse effects are usually rare (mortality rate expected below 1%). Because the genes we study are involved in very early development, the effects of gene deletions in embryos are expected to cause early embryonic lethality or sterility and so will not cause harm to any 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)?

Mild: 94%

Moderate: 6%

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 study the processes that are important for healthy embryo development, and use the mouse as a well-established and important model for mammalian embryogenesis. We need to study mouse embryos as opposed to another model because suitable stem-cell derived models (embryonic stem cells, organoids) do not fully and faithfully recapitulate the early stages of mammalian development. While we perform most of our work in stem cell lines, it is essential to explore and validate our findings in an in vivo setting to make sure that what we see relates to development and is not a cell culture artefact.

Which non-animal alternatives did you consider for use in this project?

We are actively using embryonic stem cells (ESCs) to perform pilot studies and the bulk of our mechanistic experiments, before moving to experiments in mice. For example, several of the factors we will study in mice we have shown to be essential for chromatin and/or transposon regulation in ESCs.

Why were they not suitable?

ESCs, as well as other embryo-derived lines, are important models of the embryo with many shared molecular features. However, they do not perfectly recapitulate embryonic development; for example they are unable to interact with the maternal environment in the way an embryo would, and it is impossible to study the all the reprogramming stages that occur during pre-implantation development using in vitro cell lines because it is not yet possible to recreate these transitions in a dish. Since we study the earliest stages of chromatin remodelling and gene regulation that occur in the embryo it is therefore essential to use fertilised eggs and early embryos as a model with which to study these 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?

We have estimated these numbers by performing statistical tests for the types of experiments we will do in order to calculate how many mice we will need to generate meaningful and reliable data. We have then thought carefully about how many of each type of experiment we will need to do to study the different factors and processes over the duration of the project. These numbers are based on several of our previous findings, which have been subject to peer-review and published in scientific reports. The numbers of animals have also been independently reviewed as part of successful grant applications.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animal numbers are estimated using statistical power calculations as well as using estimations of effect sizes from our previous published results. As we study pre-

implantation embryos, we will be able to take advantage of the multiple numbers of embryos per female to generate more data per mouse - for example by being able to use one batch of embryos for measuring multiple metrics at once. We have benefited recently from attending an experimental design course for in vivo scientists. Additionally, we have taken advice from senior academics at our establishment and our bioinformatics core on experimental design. For all experiments involving different conditions or treatments, embryos will be pooled from different matings and randomly separated into experimental groups to avoid litter-specific biases. Throughout the work inbred mice are used to be consistent with our previous mouse experiments and 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 stimulate females to produce more eggs prior to mating or collection, which will mean that overall fewer females are needed to provide enough eggs or embryos to study. We will continue to optimise and incorporate new low cell number techniques (such as used in our previous recent publications), meaning that smaller amounts of material are needed for experiments, which in turn will reduce the number of mice needed. Where possible, we will also rely on single-cell read-outs (such as immunofluorescence) or low-input approaches (as mentioned above) in order to minimise the number of mice required per experiment. Where possible, before larger studies are carried out for each type of experimental analysis, we will perform smaller pilot studies using fewer mice, to gain information about effect sizes and optimise 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.

Wherever possible, we will first perform experiments in embryonic stem cells (ESCs) and other stem cell lines to generate data that informs our mouse experiments and justifies generating or working with the relevant mouse models. As the majority of our research focuses on embryos, the mice we use will experience no suffering apart from temporary mild discomfort, if, for example, receiving a hormone injection. The models we use are expected to cause early embryonic lethality or infertility, which will thus not cause harm or suffering to sentient animals.

Why can’t you use animals that are less sentient?

Our work focuses on specific aspects of early development and processes that are regulated very differently between mammals and lower species, meaning that the mouse is the best model we currently have for human development, and the lowest animal model for mammalian development in general. Using mice will allow us to understand more about how early development proceeds and may therefore shed light into what goes wrong in cases of infertility. Our work on non-sentient early embryos means that adult mice only

receive temporary discomfort if given hormone injections. Otherwise, eggs and embryos are collected after terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We expect only minimal harm for the majority of animals in this project, who will suffer no or mild discomfort only, and will use enrichment of animals' environment to improve welfare. Following any surgery, animals will be monitored closely with appropriate pain relief given, in accordance with established best practice standards.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will closely follow all appropriate guidelines related to the use of animals in research, such as Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and sources such as the NC3Rs website. We receive relevant updates and recommended courses directly via email from our establishment, its committee on Replacement, Refinement and Reduction, as well as through our Transgenic Facility.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We work closely with our institute's transgenic facility and make sure we are working as effectively and humanely as possible. We receive regular communication from the National Centre for Replacement, Refinement and Reduction of Animals in Research (N3CRs) from our establishment and continue to use this and their posts on social media to stay abreast of new developments to refine animal work and maximise animal welfare. We meet together weekly as a lab group to discuss anything work related, and to allow discussion of any such advances with personal licensees. Our establishment regularly publicises relevant courses which we have strived to attend.

157. Neuronal and vascular function in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

brain, vasculature, Alzheimer's disease, pericyte, neuron

Animal types	Life stages
Mice	pregnant, adult, juvenile, 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?

How do neurons regulate their energy supply and does a dysregulation of energy supply cause disease? Brain cells require a constant energy supply of oxygen and glucose in the blood to keep them working properly. Some brain cells communicate with nearby blood vessels to increase blood flow when the brain cells are active so need more oxygen and glucose. We will investigate how patterns of brain activity modulate blood flow, how this "neurovascular coupling" is altered in disease, and whether changes in vascular function can contribute to damage in different 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 thiswork?

Elucidating how brain cells control their blood supply is important for understanding to what degree the brain's energy supply limits its function. Changes in blood vessels occur in several disease states that cause significant brain injury. For example, changes in blood vessels are increasingly thought to be important very early in Alzheimer's disease, but we don't yet understand how alterations in the brain's energy supply produce or interact with the damage to brain cells that occurs later in Alzheimer's disease. By learning more about these early changes in blood vessels that occur before brain cells are damaged, we hope to find out how to intervene to stop early vascular damage, and hopefully then prevent Alzheimer's disease from developing.

We also hope to understand how the normally-functioning blood system works, how it is regulated, and if brain cells' activity is affected by how neurovascular coupling functions in different brain regions. This will help us understand how cells might be at differently at risk from damage during disease depending, for example, on their location.

What outputs do you think you will see at the end of this project?

Outputs from this project will include publications in scientific journals communicating our findings to the academic community, as well as conference proceedings from presentations of our work while it is still ongoing. We will communicate with the general public, via outreach events, our lab website and social media platforms, as well as by writing articles and blogs for websites associated with the university and organisations with an interest in our work (e.g. the Alzheimer's Society). This will allow us to inform a wide range of people of our results. We will also generate new mouse models that can be used by other researchers studying either COVID-19 or Alzheimer's disease, and these will be made available to anyone who is interested in using them, therefore helping boost the productivity of other research groups as well.

Who or what will benefit from these outputs, and how?

Short term benefits (< 1 year) will be mostly at the academic level, whereby other researchers can use our findings to progress their own thinking and research. Such examples can include the development of collaborative grant proposals. For example, my lab's work on pericytes and neurovascular function was able to generate fast impact by enabling me to repsond to the evolving COVID-19 pandemic and realise the relevance of our work understanding the disease process. By bringing together a consortium of researchers across the fields of virology, physiology, biochemistry and clinical haematology, we were able to access rapid response funding to address the important question of which vascular cell types become infected with SARS CoV-2 - a project which I expect will bring benefits to the whole consortium in terms of productivity and proven impact.

In the medium (1-5 years) to long term (> 5 years), however, while academics will continue to benefit from our results, we hope that the public will also start to see an impact. We do frequent outreach events and the key message from our research is the link between cardiovascular and brain health. While we hope our research will, in the medium to long term, deliver novel targets for therapeutic intervention and therefore enable future treatments for dementia, and other diseases, this is not feasible in the shorter term. However, it is feasible that people could see our results and be convinced that improving their cardiovascular health is important for their future chances of getting dementia. We would therefore hope that by direct outreach, by writing articles for the general public and by adding to the academic literature supporting the link between vascular and neuronal

function, we could encourage people to improve their lifestyle, with likely medium and long term improvements to their health. In this way we would hope to benefit people who would have suffered from dementia and their would-be carers. We also hope that an understanding of the progress being made to understanding the condition (and similarly for COVID19) will be of benefit for those who do not directly benefit with an increase in health.

In the long term, we hope that all of our projects will have some impact on improving health, either by elucidating pathways and cell types to be targeted for therapeutic interventions, or by increasing our understanding of the underlying physiology so we better understand when processes begin to go wrong.

How will you look to maximise the outputs of this work?

We will maximise the effects of outputs of this work by ensuring we have engaged most fully with those who will benefit from this approach. As discussed above, collaboration is a very important part of almost all of our projects. Such collaborations maximise the success of the different projects by linking with experts who can help us to achieve ambitious experimental aims in novel directions to answer our important questions. By collaborating with others on these projects, and sharing our own expertise in other collaborations, the number and impact of our outputs will be maximised.

We will aim to publish all work, with a strategy of aiming first to publish a coherent narrative around the key research questions in high impact publications, so that there is a clear conclusion and message from each project. However, this will not be possible for all projects and we aim to publish everything, or results that we cannot fit into a clear narrative, for example because of lack of funding to fill in gaps.
This includes negative results, which could be structured around a narrative that all disproves a hypothesis (supporting the null) or that demonstrate a single, surprising result. We will also, as detailed above, aim to communicate our results widely in academic and lay circles, so that the impacts are broadly felt.

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 use mice to study how the brain controls its blood flow. Mice are the best species to use, because their neurovascular system works very similarly to our own. Secondly, lots of genetically modified mice have been created. By breeding different genetically modified mice together we can create mice that allow us to answer specific questions. For example, some of our mice express a protein that becomes more fluorescent when cells are active. We can breed mice that express this protein in neurons, muscle cells on blood vessels, or supporting cells that wrap around blood vessels. Because mice are small we can inject a small amount of dye into their veins and easily see their blood vessels very clearly, and we can fit them under a microscope to see into their brains to record their brains and blood vessels. By making these different mice, we can see how activity in different types of cells impacts on blood flow.

Another way in which these genetically modified mice are vital for these experiments, is that they enable the effects of specific disease-related proteins to be investigated. For example, we can control the expression of proteins that form clumps in Alzheimer's disease and damage neurons, to understand how damage to blood vessels may affect the rate of formation of these protein clumps, and therefore the rate of neuron damage. In most cases we will be looking at blood vessel and neuron function in young to middle-aged mice, to study processes that happen in the healthy brain or that, in mice expressing risk factors for disease, set up changes in the system that can lead to disease. In some mice, older animals may be used to model the effects of ageing on these processes.

Typically, what will be done to an animal used in your project?

Typically, a genetically modified mouse will have surgery at 3-4 months of age to implant a window into its skull. After recovering from this surgery the mouse will be gradually habituated to being held in position under a microscope on top of a treadmill, on which it can run when it chooses. When it is comfortable in the equipment, we will first inject it with a dye that will label its blood vessels and then record neurons and blood vessels in its brain and record its behaviour (e.g. running, licking) while it watches visual stimuli or runs through a virtual reality environment. Over the next 3 months, we will typically do 3-4 similar imaging sessions to measure the pattern of actiivty in neurons and how nearby blood vessels dilate, interspersed with other recording sessions where we measure brain blood flow.
The mouse might also have some memory tests outside of these sessions where, for example, we might record whether it recognises the presence of a new object in a small arena (mice explore new things more so a mouse that remembers having seen an object before spends less time exploring it). After the experiment, a mouse would typically be culled in a way that means we can preserve its brain and use this to collect more data about the anatomical organisation of blood vessels, and do experiments to label what proteins are expressed in different cells.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that undergo surgery may experience pain during recovery, though this is managed with pain killers. Mice usually recover very quickly from surgery but can be subdued for a few hours. We can treat them with more pain killers and also give them soft mash to help them recover. We monitor the animals closely after surgery to see if they need extra care. If they do not get better after giving them more care, then we cull them within 3 days of becoming ill, so they do not continue to suffer, or immediately if their suffering is severe.

Mice may experience a very short discomfort during injections that are needed to control protein expressions, or to deliver drugs or dyes into blood vessels. However this discomfort would typically last much less than a minute.

Mice may be initially stressed when getting used to being held in a head restraint under the microscope, but we accustom them to this gradually so that they only suffer a very short amount of stress (~1 min).

In a very small number of animals, we give them an injection that makes them have a flu-like illness for 24-48 h. This makes them feel poorly, but they recover within 48h. This is necessary to monitor the effects of infection on blood vessels and neurons and, in some

experiments, how COVID-19 may be affected by prior infection.

Some mice, mimicking aspects of Alzheimer's disease, may develop some memory problems but we do not expect this to impact on their welfare.

Expected severity categories and the 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: 100% mild

Experimental Protocol 2: 70% moderate (predominantly surgery), 30% mild (predominantly injection of innocuous substances and behavioural testing).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cultured cells are not appropriate for much of this work, as neurovascular cells behave very differently in culture than in the intact animal, and the spatial organisation of the cell types is lost in culture but is critical to the functioning of the system. A lot of my work is done using brain slices, where these spatial relationships are preserved, but which avoid using a living animal. I also use computer models frequently to simulate the processes underlying the observations I make from my experiments.

Nevertheless, studying the relationship between brain activity and blood flow requires that, at least for some experiments, the blood supply to the brain is intact – i.e. the animal is alive. Mice allow us to study these interactions between blood vessels and neuronal activity because they are sufficiently similar to humans to be informative about neurovascular function, there is a wide availability of genetic modifications to allow us to image cellular actiivty and to model human disease, and they are small, so more of their brain is accessible to our imaging methods than larger species.

Which non-animal alternatives did you consider for use in this project?

We use some cell culture work when this is appropriate, and use tissue from post mortem animals where possible to minimise procedures on alive animals. We also conduct computational modelling, including simulations of cell activity and nutrient diffusion to extend findings from animal tissue.
However, as described above, these approaches are not enough to inform us about the physiological interactions between the nervous and vascular systems, and how they can go wrong in disease.

Why were they not suitable?

Cells can change their nature in culture, and tissue slices do not preserve the intact vascular system, so it is important to also conduct experiments on alive animals that have nervous and vascular systems intact. Computational modelling is only informative if you have sufficient information about the system you are modelling, and can complement well-designed experiments on animals to maximise data and understanding from these 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?

We know how many mice we have used in similar experiments in my last licence. We have to date used a little under this number, so this is likely to be an overestimate rather than an underestimate, but we also have more researchers now working on the different elements of this project so may use a few more than we previously did. We will adjust numbers on the licence with amendments if it becomes apparent that our estimates are very inaccurate.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

By collecting multiple measurements from each animal, in vivo or in fixed post mortem tissue, we reduce the total number of animals needed both by maximising the information gleaned from each individual and by obtaining within-subjects data allowing us to relate e.g. vascular dysfunction, oxygenation and beta amyloid in the same animal, reducing random variation.

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 predict the numbers of mice we need to breed from so have the optimal number of breeding pairs at any one time. We also use non-experimental genotypes, where possible, to maintain our breeding colonies. We use pilot studies using only a few mice to test new experimental ideas to see what effect sizes we are likely to get, to ensure we use the optimal number of mice for detecting an expected effect, as well as to optimise data collection and minimise mice used on experiments that will not work properly. Finally, we collect a lot of data from each mouse within our lab, and are creative with the use of images and tissue collected for one experiment, so that often this may become preliminary data for another project by analysing a different element of these data to address a different question.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically-altered mice expressing proteins that label different cells, allow us to monitor and alter the function of specific cells, and/or express human proteins to mimic aspects of human disease. We use mice because there are lots of genetic tools available, but also it is possible to do the experiments we need while maintaining a good standard of welfare in mice. Mice are small, which means they can be housed in cages which give them enough space to be active and to socialise. They are able to fit under our microscopes and we can see far enough into their brains for our experiments to work. They can be handled regularly to get them used to us and the experimental process.

The genetic alterations in our mice generally do not cause them any harmful effects. The most damaging manipulation we use models some aspects of Alzheimer's disease by, at a set time point, expressing a human genetic risk factor and the human protein that is broken down to produce beta amyloid, a peptide that aggregates to damage nerve cells in human Alzheimer's disease. However, because our experiments study the onset of the disease process, even these mice will only be suffering mild memory problems at the stage when we are studying them, which we do not expect to have any welfare implications. In addition, use of this model maximises welfare compared to other experimental designs, as production of the harmful protein can be suppressed until after development, minimising its negative effects and increasing the vailidity of the model to better reflect human disease.

Many of our experiments involve performing surgery on mice to insert a small window in the skull. Mice usually recover very well from this surgery, and are grooming, eating and drinking within a few minutes of coming round from the anaesthetic. We handle the mice regularly so they are used to human contact. We then get them used to our recording apparatus very gradually, increasing time on the apparatus only when the mice are no longer stressed by it. This means that they are relaxed during our recording sessions, minimising their distress.

We have one experimental condition which is expected to cause some suffering to the animals, as it gives them an infection which makes them ill for 24-48h, similar to a bad cold. We have as few animals as possible in this condition, but we need to do these experiments as they allow us to study how infection affects the brain and vascular systems. In particular, we will be looking at how existing infection affects how blood vessels are affected by SARS CoV-2, which causes COVID-19, which is very important for understanding how this disease affects organs in our bodies outside the lung, and why some people suffer more severely than others.

Why can’t you use animals that are less sentient?

We need to use animals that are sufficiently similar to humans to be informative about how human physiology and disease work. Mice have a similarly organised brain and vascular

system and we can use measurements, such as the level of blood oxygen, that are also used in humans to compare and check the validity of our experiments for understanding humans. Our experiments on live mice are necessary to study the interactions between the nervous and vascular systems, but where possible we use other experiments on cultured cells, or brain tissue from mice that are no longer alive, to minimise the level of any harm, as well as computer simulations. These experiments are good for learning about specific signalling pathways, and how simple systems work, but we need the experiments on more complex systems to understand how these simple systems interact in real life.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We already continually refine our methods to minimise welfare costs, so are already doing the level of monitoring and post-operative care that has been indicated from our previous experiments.

Refinements in the course of my previous licence have included altering the cranial window implanatation standard operating procedure and training to highlight the importance of scoring the skull before gluing on the head plate, to enable better adherence of the implant to the skull, and increased monitoring and early interventions in potentially loose implantations to detect and correct any problems with the implant before these become serious. We have also altered the regime under which we perform experimental recordings, increasing the number but on average reducing the duration of these recordings. This increases the degree to which mice are habituated to the equipment while also reducing the duration of head restraint, so they can groom and engage in natural behaviours more frequently. When making this change we conducted empirical observations of welfare to verify our intuitions that these changes would not negatively impact welfare. The methods described in this licence application therefore include all our current refinements, but we consider this process of iteratively considering our methods and whether welfare can be improved through further refinements to be a key part of our experimental methods and duty of responsibility when conducting experiments on live animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use a range of resources, including academic papers using similar methods or mouse lines. For example, Sri et al (2019; PMID: 30795807) demonstrate that developmental expression of APP in the tet-offAPP/tTA inducible mouse line causes changes in brain development that we want to avoid for both welfare and scientific reasons. This publication therefore leads us to refine our expeirmental design and provide doxycycline in the diet of mice from before birth. We also refer to resources and publications provided by organisations including NC3Rs and LASA, including the LASA/RSPCA report on avoiding animal mortality, the LASA guide to aseptic technique, the NC3Rs newsletter and the ARRIVE 2.0 guidelines for reporting of animal experiments.

Other useful resources we use include Mousewelfareterms.org which provides a list of terms that are useful for assessing and decribing mouse welfare, and the information provided by vendors of genetically altered mouse lines to plan for known phenotypic effects found in those strains (e.g. references and information on www.jax.org).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances and refinement of our methods is always an ongoing process. We receive regular email updates from the Laboratory Animal Science Association (LASA) and the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) which both contain useful information about training and new approaches that may be of use. We have regular meetings with our Biological Research Facility who are also able to suggest refinements. Knowing about advances in other research groups is useful, but it is by attending and thinking critically about our own experiments, however, that we are best able to quickly adopt refinements to improve welfare druing our projects. For example, we have hugely refined our surgical methods and post-surgical monitoring during the course of my last project licence, as we identified when things were working less well, gathered advice from other researchers, the Named Animal Care and Welfare Officer and local veterinarian and were able to iteratively improve components of our procedures to improve the surgery and early identification of post- surgical complications. We have now shared this good practice with other groups to allow them to adopt these advances themselves.

158. Role of cellular and molecular therapies in liver injury

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.

Key words

Liver, Stem cell, Inflammation, Liver fibrosis, Trafficking

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

There is a large clinical unmet need in liver disease, for which cell therapy offers exciting new possibilities. Cell therapy has real potential to help reduce scarring in liver disease, reduce ongoing liver inflammation and also contribute to replacing liver cells. These studies will explore ways of enhancing our current knowledge by applying cell therapies to more clinically relevant models as a prelude to clinical studies. In addition these studies will look at ways of enhancing the action of cells by improving their action and delivery to the injured liver.

Advanced therapies/Cell therapies will be studied that have the following actions:

Reducing liver scarring

Haematopoietic stem cells/mesenchymal stromal cells will be used as they have been shown to reduce scarring in mouse models and also in clinical studies.

Reducing liver inflammation (ongoing damage)

Mesenchymal stromal cells/related material and evasins will be used as they have been shown to reduce inflammation in mouse models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

What are the potential benefits that will derive from this project?

This project will provide important data that improve the action of cell therapy alongside our understanding of how cells exert their benefits. This will underpin new clinical trial submissions to the MHRA. Evidence of action and safety of cell therapy from appropriate mouse models will be a key part of submission to the MHRA.

As well as leading to the generation of new clinical therapies this will also provide insights into the mechanisms of liver damage. This in turn will drive additional new therapeutic options which may use drugs rather than cell therapies.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

5000 mice will be used in total over a 5 year period, of which genetically altered mice will be used to more closely represent the clinical condition seen in patients. This will include models that recreate liver inflammation (acute carbon tetrachloride, Ova-Bil and MDR2 ko) and liver fibrosis (chronic carbon tetrachloride, TAA and MDR2 ko).These models are widely regarded in the scientific community as being the closestmodels to the situation seen clinically.

The number of mice used will be based on statistical power calculations (80%) as used in clinical trials. Based on the anticipated benefit from a cell therapy and the variation in the parameter being measured, we will calculate how many mice are needed to provide a statistically robust answer. We have in-house access to statisticians as well as our own experience to ensure that these calculations are accurately performed.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Models have been selected that closely mimic the varied clinical scenarios seen in patients. The models selected are commonly used and are deemed representative in the scientific and regulatory community. These models develop liver injury but do not demonstrate any sign of suffering.
Suffering will be reduced/stopped by the continued adoption of our unit guidelines. These ensure that side-effects are looked for and when found injury is either discontinued or the experiments are discontinued.
Routes of cell administration will for the vast majority of studies be by ways which do not cause any discernible harm in our experience. Only rarely will surgical routes of

administration such as intra- splenic or intra-hepatic routes be used, and this will largely be reserved for infusion of liver-like cells in keeping with the literature and clinical practice.

Repeated general anaesthesia will only be used when non-invasively imaging the distribution of infused cells in mice. This reduces the number of mice that might otherwise be used. Modern anaesthetic agents will be used which shorten recovery times for mice.

Moderate severity is based on the clinical outcomes seen with the models outlined in this licence. This is in keeping with our previous experience over the preceding two licences.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

The actions of therapies are complex often involving a complex interplay with a variety of cells that cannot be assessed in in vitro assays. This applies equally to human tissue as it does to mouse tissues used in in vitro settings.

We will use in vitro experiments to reduce the number of studies required although it does not capture the nuances around their efficacy in vivo. Ex vivo studies with human liver cells will provide unique data on the anti-inflammatory effects of cell therapies on endothelial cell recruitment.

Reduction

Explain how you will assure the use of minimum numbers of animals.

Use of inbred mice reduces variability and hence the number of experiments required. The number of mice in each experiment will vary according to the specific model, and the anticipated difference expected.

As we now have experience of the effect seen with our cellular therapies the numbers of mice needed can be informed by the use of statistics. Numbers are also based on studies in my previous project licence, and will be influenced by the variation in the parameters being assessed. In that regard the number of mice can vary from 3-4 in each arm up to 20 in each arm depending on the parameter being tested.

Variability will be controlled by using mice of the same gender of a similar age and weight.

We ensure that any tissues generated from previous experiments are archived and stored appropriately therefore ensuring that unnecessary repetition of experiments is not necessary.
We will aim to use the NC3R's experimental design assistant to aid experimental design and report the work in accordance with the ARRIVE guidelines when reporting for publications.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take

to minimise welfare costs (harms) to the animals.

Models have been selected that closely mimic the varied clinical scenarios seen in patients. The animal facility has experience and expertise in all these models.

Suffering will be reduced by the continued adoption of current best practice. Mice are closely monitored for signs of adverse effects; from experience humane end points have seldom been reached. Where at all possible mice will be killed by Schedule 1 humane methods of killing.

Cells and substances administered will be at volumes and frequencies such that they will cause no more than transient pain and distress and no lasting harm, in accordance with published guidance.

Repeated general anaesthesia will only be used when non-invasively imaging the distribution of infused cells in mice. This reduces the number of mice that might otherwise be used. Modern anaesthetic agents will be used which shorten recovery times for mice. Routes of cell administration will be the most refined whilst scientifically justified and from experience do not cause any discernible harm. These include intravenous, intraperitoneal and subcutaneous routes.

Moderate severity is based on the clinical outcomes seen with the models outlined in this licence. This is in keeping with my previous experience over the preceding two licences

159. Mechanisms of real-time motor learning in the cerebellum

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

brain, movement, learning, neural networks, neurons

Animal types	Life stages
Mice	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?

To advance understanding of how the cerebellum coordinates movement and behavioural responses triggered by learning.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The cerebellum forms a large part of the brain and is important in coordinating movement and the formation of learnt associations. Understanding of how neurons in the cerebellum function during normal daily activities will provide important insights into how the brain processes information. This basic level of understanding is an essential first step in the development of brain-machine interfaces (e.g. motor prostheses). The data generated will be of value to scientists and developers working in the field of artificial intelligence, robotics and neuro-informatics. In the long term, the finding of this and other studies are expected to feed into the development of new approaches for treating movement and plasticity-based disorders of the central nervous system such as occur during a stroke, traumatic injury or tumour.

What outputs do you think you will see at the end of this project?

Knowledge resulting from the project will be of scientific, clinical and economical value. We will generate new publications and datasets that will enable information about cerebellar function to be linked up with research in other brain areas, including the cerebral cortex. We will generate a new quantitative model of a fundamental model brain circuit, and this will enable in silico exploration of the learning processes that take place in complex behaviours including multi-limb body movements, and decision making. More broadly, we will provide new information about how the brain learns and processes information. This is essential information for the development of new brain-machine interfaces and will be of value to developers working in the field of artificial intelligence, robotics and neuro-informatics. Findings from our studies may help the development of new approaches for treating clinical problems such as stroke, dementia and brain injury.

Who or what will benefit from these outputs, and how?

We will provide biomedical researchers, engineers and clinicians with valuable information to help them develop brain-computer interfaces. Biomedical researchers will benefit immediately from our outputs upon dissemination. Engineers stand to gain by using our findings to constrain computer models using biological principles. This benefit will take place 1-3 years following dissemination. Our findings will ultimately guide development and application of new therapies in the clinic. The expectation is that these benefits will be realised 5-10 years following dissemination.

How will you look to maximise the outputs of this work?

We collaborate directly with computational scientists who use our data to develop mathematical models of the brain. We will publish our work in journals and also online on relevant preprint servers (e.g. bioarXiv). We will ensure all data will be made freely available to researchers in the field following publication. We will communicate our work directly at public events, presenting short talks to describe the work that is being performed in the laboratory in an accessible manner. All together, we generate enthusiasm and understanding for the work we are undertaking, and more broadly, basic neuroscience research.

Species and numbers of animals expected to be used

- Mice: 1850

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice as these animals have similar brains to humans and can be trained to perform learning and memory tasks. Genetically altered mice also enable us to record and control the activity of cells in the brain. This enables us to determine the role of different brain circuits.

Typically, what will be done to an animal used in your project?

Each animal undergoes general anaesthesia (lasting 2-3 hours), where they receive injections into the brain and/or an implant onto the skull. After a recovery period of at least 3 days, animals undergo training motivated using water rewards. The typical length of this process is 4 days of familiarisation followed by 21 days of task training. Then animals undergo 5 daily sessions of brain recording. Each session lasts approximately 1 hour. At the end of the study, animals are anaesthetised and killed. The maximum study length is 3 months inclusive of 29 days of behavioural testing.

What are the expected impacts and/or adverse effects for the animals during your project?

Animal fails to recover fully from surgical procedure (incidence: < 2%).

Head implant becomes displaced (incidence: < 2%). Duration 24 hours max.

Weight loss due to water regulation (incidence: < 5%). Duration 24 hours max.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Non-recovery (10%)

Mild (15%)

Moderate (75%)

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?

Critical gaps in current understanding of how the brain functions include how brain cells and the connections between them change during learning to create memories and coordinate movements. It is not possible to address these gaps without studying these processes in living conscious animals as they perform natural behavioural tasks. In order to ensure that the study findings are relevant to humans, it is necessary to use mammals for these studies.

Which non-animal alternatives did you consider for use in this project?

We considered (1) computer modelling and (2) recordings from cultured neurons as

alternatives. However, neither approach provides a suitable alternative to the use of animals.

Why were they not suitable?

(1) Computer modelling requires biological data to build models and make accurate predictions. (2) Cell cultures do not respond in the same way as living conscious brains. Non-animal alternatives are unsuitable because we need to access information about brain activity in living, behaving 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?

We have reached this estimate based on several assumptions:

Intracellular recordings (Objectives 1 & 3): we will measure how brain connections change before and after learning. A sample size of 11 is assumed for each condition, therefore 22 animals are necessary to detect significance. Comparisons will be performed for 6 different cell types, requiring a subtotal of 132 (22 x 6) mice. Cell-type specific comparisons will be done in three different mouse strains requiring 396 (3 x 132) mice. To assess pairwise changes before and after learning, comparisons for 6 cell types cannot be done in the same animals.

Subtotal: 396 animals

Large scale extracellular recordings (Objective 2): we will measure the strength of brain signals in 3 different conditions. We assume a standard deviation of 0.5 and a change from 1.0 to 1.25 in the mean between different conditions. We estimate 11 animals per condition for sufficient power to compare between groups. We will measure activity with 6 different perturbations. We will record in 3 different brain regions. All conditions/regions/perturbations need to be independently sampled due to technical constraints. Therefore 3 (conditions) x 6 (perturbations) x 3 (regions) x 11 (sample size) will require 594 mice.

Subtotal: 594 animals

An attrition rate of 10% is assumed for the above categories to account for animals that do not learn the behaviour.

Subtotal: 99 animals

Other procedures: approximately 120 mice will be necessary for training and tissue preparation.

Subtotal: 121 animals

In addition, 640 animals will be necessary from breeding of different mouse strains.

Subtotal: 640 animals

TOTAL: 1850 animals

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used an online tool to perform power calculations (http://www.dssresearch.com/toolkit/sscalc/size_a2.asp). Following consultation, we assumed a standard deviation of 0.5 and a change from 1.0 to 1.25 in the mean between different conditions.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We record from large numbers of brain cells at the same time – reducing the overall number of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

These studies use mice that have undergone procedures to enable the response of the brain to specific cues to be monitored. They undertake normal behavioural activities in order to study how movements and memories are stored. It is critical to these studies that animals are not impaired in any way. We use anaesthesia, analgesia, and full aseptic precautions to ensure that any pain caused by surgery is minimised. Animals are constantly and closely supervised by trained individuals and advice sought from the veterinary team if there is any cause for concern.

Why can’t you use animals that are less sentient?

Mice are the least sentient species whose brain function closely resembles that of humans. They can be easily trained to carry out recordable tasks and are amenable to the genetic technologies needed to facilitate the investigations. This work cannot be carried out without recording from the brains of live animals as they are performing real behaviours, therefore consciousness is essential.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimize suffering, all procedures that involve pain, such as surgery, will be performed under anaesthesia and post-surgical analgesia will be given. Animals will be monitored for signs of distress or weight loss, and will immediately be removed from the study if these are detected. For experiments in which conscious animals are necessary, we carefully monitor them throughout all sessions. If animal are not comfortable, sessions are cancelled. We have previously refined our water regulation procedures to improve task motivation (reducing training times and animal numbers). In our training sessions, mice receive a daily opportunity to run and partake in mentally stimulating activities.

We will consult with NVS/NACWO throughout the duration of the project for ongoing management, and will review our refinement procedures after 12 months.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 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 part of a working group of international researchers and animal welfare experts. We are compiling the results of the global survey on water restriction and head-fixation and are drafting recommendations for good practice. We will adopt all of the recommendations. I am also part of a group that disseminates best practices at my host institution. We will share refinements with our peers via this forum.

160. The pathophysiology of endometrial 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

K**ey words**

Endometriosis, Recurrent pregnancy loss, Macrophage, Pain, Therapy

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.
What's the aim of this project?

To determine mechanisms underpinning endometrial disorders with a specific focus on recurrent pregnancy loss and endometriosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 endometrium is a complex, dynamic tissue vital for reproduction. It is also the origin of a number of disorders that significantly impact the health-related quality of life of women. For example, recurrent pregnancy loss impacts 1-2% of all women and causes significant emotional trauma. Endometriosis affects 190 million women worldwide, and is associated with debilitating pelvic pain and infertility.
Current therapeutic options are extremely limited and have significant drawbacks. The mechanisms underpinning the pathophysiology of these disorders are not well understood and further *in vivo* work is required to identify how we can develop better treatments.
What outputs do you think you will see at the end of this project?

We plan to produce data that demonstrates significant advancement into the understanding of the endometrial disorders; recurrent pregnancy loss and endometriosis. The outputs will include peer reviewed publications in cross-discipline journals (publications produced in accordance with ARRIVE guidelines), book chapters, review articles, conference presentations. We hope that our work will eventually lead to the development of new therapies for endometrial disorders.

Who or what will benefit from these outputs, and how?

The short-term benefits of this work will be mechanistic insight into the pathological processes involved in endometriosis. The key advantage of using animals is the maintenance of the complex tissue architecture of the endometrium and the ability to model key processes of endometrial signalling during implantation, menstruation and development of endometriosis lesions in the peritoneal cavity. The long- term benefits of these studies will be the application of the insight gained from the mechanistic studies to the development of medical therapies for endometriosis which impacts the health of 190 million women worldwide. We envisage that these therapies will improve wellbeing by alleviating chronic pain and restoring fertility.

How will you look to maximise the outputs of this work?

Discussion with clinical colleagues and patient groups has informed the direction of our research effort. The translation of our findings for patient benefit will be assured by conducting studies in close collaboration with clinicians, validation of findings in human samples and by fostering links with pharma and biotech companies. The findings generated during this program of work will likely apply to other chronic inflammatory disorders, thus increasing the reach of our research. We will disseminate our findings, both positive and negative results, in publications and conference presentations.

Species and numbers of animals expected to be used

- Mice: Approx 3000 experimental animals in total (excluding animals produced via Breeding). Mice will be bred in house to produce transgenic experimental mice. We will produce around 2500- 3000 transgenic mice, but only half of these will be used in the Protocols detailed in this PPL (we only use female mice). Approx. 50% of the animals used in this study will be transgenic (approx. 1500).

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using female (young adult approx. 8 weeks of age) wild-type and genetically modified mice to investigate the pathology of endometrial disorders including recurrent pregnancy loss and endometriosis. These disorders occur only in females and we are using young adult female mice to avoid maintaining animals unnecessarily.

Typically, what will be done to an animal used in your project?

We will perform surgical procedures, injection of hormones and drugs and mating of females with males. Certain protocols require multiple surgeries in order to achieve the desired end result e.g. endometrial shedding akin to menstruation. Some experiments may last for up to 6 weeks, whilst others will be relatively short (1 week). At the end of the Protocols animals will be euthanized. One exception to this is at the end of the endometriosis Protocol, where some animals (approx. 2%) will be transferred to another PPL for additional testing prior to being euthanized.

What are the expected impacts and/or adverse effects for the animals during your project?

Endometriosis is thought to develop due to the spread of fragments of the lining of the womb into the pelvic cavity during a phenomenon known as ‘retrograde menstruation’. Mice do not usually menstruate or develop endometriosis but by administering hormones they can be stimulated to undergo a process very similar to human menstruation. This material can be collected and used to induce endometriosis by introducing it into the pelvic cavity of recipient mice. The animals show no visible signs of pain or distress during bleeding or endometriosis development, but some changes in their behaviour to certain stimuli can be detected. These changes in sensory behaviour can be detected from 3 weeks post tissue injection. Most animals are not maintained beyond this stage. New treatments for endometriosis can be identified and tested in the endometriosis model. An intervention will be discontinued if the mice show any visible signs of pain or distress that cannot be controlled with pain relief. At the end of experiments mice will be euthanized or continued onto another PPL to enable visceral response testing (approx 2% of mice).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the lines of GA mice that we will be breeding and maintaining we expect that 99% of mice will have a sub-threshold / mild severity rating.

For the remaining protocols we expect that 74% of animals will have a moderate severity rating because the mice will experience multiple surgeries and injection of hormones and / or drugs. We expect that 25% of mice will have a mild severity rating, this accounts for sham and naive controls or those that undergo limited manipulations.

Overall, potentially 1% may exceed the moderate 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?

The murine models are necessary as the endometrium is a dynamic, hormone responsive, multicellular tissue. Complex endocrine, vascular, neuronal and immune interactions necessitate an *in vivo* model that mirrors key process occurring in women: menses, embryo implantation and the attachment of retrogradely shed endometrium within the peritoneal cavity. Lower organisms such as flies and frogs do not have a uterus. Experiments carried out on *in vivo* animal models will be limited to those questions that cannot be answered using other methods.

Which non-animal alternatives did you consider for use in this project?

Our previous work has used human cells and tissues; human embryonic stem cell derived sensory neurons, human peripheral blood monocyte derived macrophages, human endometrium, peritoneum, and endometriosis lesion biopsies from patients. I will continue to use these methods wherever possible. The results have allowed us to develop focussed experiments using our mouse models that are already established and current data is available. I have attended the FRAME training school and consulted the NC3Rs websites to ensure best use of our *in vivo* models, we have also used PREPARE guidelines to assist experimental design.

Why were they not suitable?

We still use *in vitro* alternatives but some of our specific research questions require *in vivo* experimentation. For example we have used human embryonic stem cell derived sensory neurons to explore macrophage-nerve interactions *in vitro*. We will also be using macrophage cell lines for some work. We also plan to implement human organoids in the lab.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 derived from extensive use of the models previously and an in-depth knowledge of the minimum numbers of mice required to achieve statistically robust data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Breeding of transgenic mice will be kept to a minimum by sharing resources and monitoring by maintaining databases of all results. Statistical tests have been performed to increase the precision of our experimental data and limit the number of animals used. Careful experimental design will also limit numbers; we will utilise a randomised design wherever possible. Inbred strains of mice will be used where possible to limit genetic variables. Group sizes will be minimised for transgenic knockout models by use of littermate controls to minimise inter-animal variability.

To minimise experimental bias the study will use a randomised design where experimental units are allocated to treatments at random. Mice will be ear-notched to allow randomisation and identification. Animals will not need to be housed individually at any point because they can be identified by ear notch. Behavioural testing and tissue recovery will be performed in a blinded fashion.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In vivo studies will not be conducted in isolation but will build on information obtained from investigation using in vitro primary cells and cell lines and ex vivo tissues. Data mining and bioinformatics will complement all studies to ensure results do not unnecessarily duplicate published work. Findings will be published in peer-reviewed academic journals.

Non-invasive bioluminescent imaging will be used as a reduction strategy as it allows collection of data from the same animal over several time-points and reduces variability.

We have used the 'menses' and endometriosis models for approx. 8 years now and are familiar with how many mice we can proficiently handle at one time and how many mice are required to observe an endpoint that is biologically significant e.g gene expression, difference in number of macrophages, significant difference in sensory behaviour, different in number or size of endometriosis lesions. We are also experienced with ensuring that as many endpoints can be assessed per mouse as possible to avoid repeating experiments unnecessarily. e.g. collecting peritoneum, uterus, lesions, peritoneal fluid, nervous tissue from the same mouse (and performing behaviour assessments).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using mice to model endometrial pathologies. The methods we are using to model endometrial disorders use the least number of procedures as possible. For example we have recently implemented a minimally invasive model of endometriosis and we will endeavour to use this model wherever possible.

Why can’t you use animals that are less sentient?

Lower experimental organisms such as flies, frogs or fish do not have a womb. Mice have a similar womb structure to women that responds to hormones so we can stimulate it to undergo a process like menstruation and investigate the process of embryo implantation. Uterine material is also collected and introduced into the pelvic cavity of recipient mice to model endometriosis. This model of endometriosis more closely mirrors the process by which endometriosis develops in women and is a significant Refinement on previously published models (we have also implemented non-invasive bioluminescent imaging of

endometriosis lesions allowing multiple recordings from a single mouse). Mice with genetic alterations will specifically allow us to investigate the role that different cells (e.g. macrophages) and genes play in the disorder.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice will be administered pain relief before any surgical procedures to minimise suffering. Anaesthesia will be used for surgical procedures. Surgeries that will result in moderate severity include removal of ovaries and injection of womb lining into the pelvic cavity to induce endometriosis. Animals will be housed in accordance with UK Home Office guidance to maximise their environmental welfare.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs, PREPARE and ARRIVE and LASA good practice guidelines for adminstration of substances.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By attending NC3Rs webinars, local conferences and efficient communication with staff at the Unit. We will endeavour to implement any advances once we are aware of them.

161. Generation, breeding and maintenance of genetically altered rodents

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Physiology, Genetic Manipulation, mice

Animal types	Life stages
Mice	adult, embryo, pregnant, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The creation, breeding and supply of novel and established lines of Genetically Altered Animals(GAA). A GAA is a mouse that has had its genome altered through the use of genetic engineering techniques. Genetically Altered mice are commonly used for research as animal models of human diseases, and are also used for research on genes. Mice will be of high health status and defined genetic quality for use in research projects at this and other establishments. Cryopreservation of such lines to provide health and genetic security. Mouse eggs or sperm are frozen in liquid Nitrogen, to avoid loss by contamination, genetic drift or unexpected disasters eg fire in a facility.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 recognised how important genetically altered mice are in understanding the pathophysiology of many disease conditions including cancers, cardiac and metabolic diseases, as well as improving our knowledge of basic physiology. Creating, breeding and maintaining genetically modified animals we will ensure that such animals generated are

produced to the highest standards of health and welfare enabling more reproducible and publishable research. Thus we are able to recruit top quality research scientists. The animal unit has the expertise in creating, breeding and husbandry of such animals to be in full control of the breeding programmes providing a direct welfare benefit and is administratively efficient to ensure minimum wastage, allowing several research programmes to use the same animal line, breeding to project requirements, all within a specified time frame.

Cryopreservation provides security against genetic drift and health status contamination whilst meeting the 3R's.

What outputs do you think you will see at the end of this project?

Provision of GA models to researchers, resulting in new knowledge of physiological and disease mechanisms and dissemination via peer reviewed publications

Who or what will benefit from these outputs, and how?

Researchers here and their collaborators, other research facilities who do not have the technical expertise to create GA mice.

How will you look to maximise the outputs of this work?

Peer reviewed publication of the subsequent work on these animals, including encouraging publication of any failures of the approach, collaborations with other institutions.

Species and numbers of animals expected to be used

- Mice: 53700

Predicted harms
Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are a well recognised species for work involving genetic alterations and there are standard protocols, methods and reagents used that have been optimised for this species and acknowledged benefits for use.

Typically, what will be done to an animal used in your project?

The majority of the animals will be used for the breeding and maintenance of genetically altered or mutant animals. Some animals will undergo surgery as follows-

Typically female mice will be mated with vasectomised (Sterile) male mice to induce pseudo- pregnancy(In mammalian species, pseudo-pregnancy is a physical state whereby all the signs and symptoms of pregnancy are exhibited, with the exception of the presence of a foetus, creating a false pregnancy). They will then undergo a minor surgical procedure, under general anaesthesia, to implant previously genetically modified embryos into the oviduct. During this procedure analgesia will be administered. The females will be

allowed to recover and give birth. Aseptic technique will be observed throughout. The resulting offspring will have a small piece of ear tissue removed to confirm the genotype.

What are the expected impacts and/or adverse effects for the animals during your project?

superovulation-There may be momentary pain on 2 occasions when an I. P, an injection into the abdomen, is administered.

Recipients- A female mouse will have a small, less than 1 cm incision, to expose the Uterus, enabling delivery of embryo's into the uterus. Mice recover rapidly and, in our hands, without complications.
Anaesthesia, analgesia and aseptic technique used to prevent pain and infection.

vasectomy-Minor surgery from which mice recover rapidly and, in our hands, without complications. A small incision approximately 5mm, is made into the scrotum and the Vas deferens is cut to render the mouse infertile. Anaesthesia, analgesia and aseptic technique used to prevent pain and infection.

Breeding of genetically altered animals with mild phenotype (the observable traits eg eye colour is a phenotype). These animals are not expected to show any deviation from normal wild type mice. A typical breeding female will have 6-8 litters in her lifetime.

Administration of a transgene inducing or deleting agent may cause minor pain during injection and weight loss as a result of the action of inducing or deleting a gene. Some mice have a gene which is inactive until the mouse is given one of these activating drugs.

Expected severity categories and the 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, approximately 90%, of the mice are perfectly normal and will suffer severity which is sub- threshold. Approximately 5% of the mice will have 2 injections into the abdomen which is mild severity. Some mice, approximately 1%, will undergo minor surgery will have moderate suffering which is expected to be brief and resolve without complication.

A small proportion of the mice will be bred with an altered immune system meaning they could be prone to infection. we will use barriered housing and sterile food and water plus aseptic handling to reduce this risk.

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?

New technologies are improving the field of animal transgenesis and they will allow the generation of new mouse models to be applied in biomedical research. First steps in a scientific project will involve in vitro approaches, the final characterisations and applications will require 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 processes

Which non-animal alternatives did you consider for use in this project?

We have an established cell engineering service as a potential alternative to in vivo models in vertebrates.

Why were they not suitable?

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.

Invertebrates are a useful adjunct to animal studies in that large throughput of gene alterations can be screened but the differences in circulatory, neurological systems etc limit their use.

Prior to importing or creating a new strain, consideration will be given to the scientific evidence gathered from in vitro data e.g. receptor binding assays to identify appropriate targets and PCR analysis of gene expression. The breeding method and proportion of affected animals produced will also be considered.

All of these factors will be used to justify the introduction and creation of strains under this licence with advice and discussions taken with the researcher and NVS (Named Veterinary Surgeon) as 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?

These numbers are predicted, based on analysis of the number of animals used in the last five years to meet the demand of our researchers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Switching to an IVF (In Vitro Fertilization) -based approach to generate mouse embryos in place of traditional overnight mating's reducing the number of mice used by half.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Using efficient breeding techniques including use the Home Office GAA breeding guidelines

Switching to an IVF-based approach to generate mouse embryos in place of traditional overnight matings reducing the number of mice used by half

Integrating CRISPR-Cas9, a gene editing technology, which has greatly reduced the numbers of mice required to make a new transgenic strain for many allele types.

Developed our own method to generate high efficiency long single stranded DNA (lssDNA) donors for gene knock in 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.

This service licence will be used to breed and supply genetically altered mice for research.

Mice are a well-recognised species for work involving genetic alterations and there are standard protocols, methods and reagents used that have been optimised for this species and there acknowledged benefits for use.

Embryos, gametes, sperm and ovarian tissue will be collected from donor strains. They may be cryopreserved or used for the following purposes.

Fresh or frozen embryos and sperm will be used for rederivation of infected mouse strains to improve health status.

Gametes, embryos/sperm or/and tissue will be archived by cryopreservation for strain storage in support of the breeding colonies.

Gametes, embryos/sperm or/and tissue will be used to replace a strain for storage where there is no longer a research requirement to avoid wastage from maintaining ‘tick over’ breeding programmes.

Gametes, embryos/sperm or/and tissue will be used where possible instead of live animals to transfer strains to other establishments with in the United Kingdom and abroad

Why can’t you use animals that are less sentient?

It is fundamental that the GAA embryos are implanted into a receptive female and she is then allowed to complete gestation. The resulting offspring will be used in research projects for which the whole mammalian body systems are required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

One of the chief benefits of undertaking this work under a service licence is that all the techniques to be used are undertaken by a small group of highly experienced technical staff which minimises suffering. It also ensures that the highest standards of asepsis are maintained and that appropriate and effective analgesia is always used.

At present NSET represents 5% of our embryo transfer procedures but we aim to increase this proportion within the lifetime of this licence. This is a process where the embryos are introduced into the uterus via the vaginal route rather than surgical implantation. At present this is not as reliable in terms of number of pups born as the surgical method.

We are a part of an NC3R's funded project to develop a genetically sterile male mouse which could replace the vasectomy surgery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will consult with colleagues across the field and sources such as AWERB
International society for Transgenic Technologies Animal Welfare and Management Discussion Group NC3R's Efficient Breeding Strategy
Institute of Animal Technology

Efficient Breeding of Genetically Altered Animals Assessment Framework(Home Office)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advice has been sought from NC3R's project manager on the development of this PPL application and will continue to be sought during visits this facility on a weekly basis.

162. Understanding vision and developing therapies for blindness

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

vision, retinal degeneration, neuroscience, circadian rhythms, blindness

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo
Rhabdomys pumillio	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 eye allows us to see. It also drives a number of sub-conscious responses to changes in our light environment (collectively known as 'non-image forming responses) such as resetting the phase of our biological clocks and keeping us awake. We aim to understand how the eye and brain work together to allow us to see and to support 'non-image forming' responses to light. We also determine new ways to manipulate cell activity using light in the search for ways of restoring vision in the blind.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

Vision is one of our most valued senses and impairments in vision reduce quality of life.

Many of the most common types of visual impairment are currently untreatable. A more detailed understanding of how vision works, holds the promise of better appreciating disease conditions and developing new therapies. We will also use our knowledge of mechanisms of light detection to explore ways of making cells light responsive and apply this to developing new approaches to treating currently incurable forms of blindness.

A particular interest of our work is such 'non-image forming' responses to visual stimuli. Light can regulate practically all body systems either directly or by its impact on our internal (circadian) body clock. We know that disrupting this control can lead to widespread and intractable public health problems such as obesity and mood disorders. Understanding how vision drives these responses has led us to better control our light environment to support human and animal health.

What outputs do you think you will see at the end of this project?

We will achieve a deeper understanding of how the retina and brain allow us to see, what goes wrong during blindness, and how vision may be restored. We will also improve our knowledge of how light influences mammalian circadian rhythms, and behavioural and physiological state. We will publish our work in scientific papers and make our data available for others to use in their studies. We hope also to use our findings as a starting point for clinical trials of new therapies in patients.

Who or what will benefit from these outputs, and how?

Our work will benefit people suffering from retinal degeneration (by some estimates 1 in 3500 people in industrialised societies). It may also benefit a much larger fraction of the human population by providing a greater understanding of how light influences our biology, which can be used to improve design of architectural lighting to better support human health.

How will you look to maximise the outputs of this work?

We have active collaborations with the lighting and consumer electronics industries to see our insights translated into new processes and devices. We also provide guidance to lighting standards and regulatory bodies around the world to ensure that built environments provide the best support for health and wellbeing.

We have ongoing collaborations with the biopharmaceutical industry to see our discoveries in the field of retinal degeneration translated to patient benefit (including a clinical trial in preparation).

Species and numbers of animals expected to be used

- Mice: 7800
- 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.

Most experiments will be on mice. We have chosen these animals because we already know a great deal about how mouse vision works and this will make it easy to interpret the outcome of our experiments. Mice have a visual system that is designed to work well under dim light allowing us to understand that aspect of human vision. Finally, mice naturally have many of the genetic mutations that cause blindness in humans, allowing us to use mice to study human disease. One limitation of mice is that they do not have such good vision in daytime conditions. In order to get a full answer to these questions we will therefore also include a day active species with good bright light vision. We have chosen to use the African striped mouse (Rhabdomys) that has good daytime vision. The rodent visual system develops post-natally. We will therefore work on adult animals.

Typically, what will be done to an animal used in your project?

The most common experiment will be to present an animal with one or more visual stimuli (anything from a single brief light flash to a change in the colour/intensity of the light in its home environment lasting several days or weeks), and measure the animal's response. The response could be a change in the animal's pattern of activity, pupil constriction, or performance of a task to receive a food reward. It may also be a change in the electical activity of the retina or brain. In some cases these methods of measuring vision will be applied to animals which have inherited problems with vision to study human disease, or animal models of neurodegeneration like Alzheimer’s, and we also may use techniques of gene therapy (injection of gene vectors) or pharmacology (application of drugs) to change the function of visual neurones and/or as a method of looking for new treatments for humans. Animals may be anaesthetised to record retina/brain activity and for injection of gene vectors.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgery will cause pain during the recovery period, this will be treated with pain killers and we do not expect it to last for more than a few hours. In rare cases an injury to the tail may appear some days after surgery, this will be treated with pain relief ensuring discomfort lasts no more than a few hours. There will also be transient stress associated with handling for injections and when animals are placed in unfamiliar environments for some of the ways we record behaviour. Some animals will be put on restricted food for several weeks in order to motivate them to seek a food reward during training based visual discrimination tasks, the level of feeding is set such that it does not impact general health or wellbeing. Some animals will be housed under non-24h cycles of light and dark for several weeks, which may theoreticlly result in discomfort but does not in practice produce signs of reductions in general health or welfare. Some animals will have inherited blindness, this does not produce signs of suffering in mice. Some animals will be Alzheimer’s disease (AD) models, phenotypic consequences of these models will be of at most mild severity.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

20% animals will experience sub-threshold severity. A further 50% will experience mild

suffering, and 30% moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are interested in how the visual system functions in health and disease, and in the utility of gene- based therapies for retinal dysfunction. Vision is an emergent property of the retina and the brain and, as such, it can only be studied using humans or animals.

Which non-animal alternatives did you consider for use in this project?

We considered using computer-based simulations of the visual system, human volunteers, stem cell derived retinal 'organoids' and neurones, and immortalised cell lines. We also considered working with tissue collected from animals.

Why were they not suitable?

We do undertake experiments on human volunteers wherever possible. We also have developed methods of recreating light sensitivity in cell lines in the laboratory and use these extensively as an alternative to animal experiments. We use computer simulations of the visual system to better understand our findings and to generate testable hypotheses. Wherever possible we use tissue collected from animals. However, there are important reasons why none of these approaches can replace animal experiments. Vision and circadian light responses are produced by multiple regions of the retina and brain working together. We are a long way from being able to recreate such a complex system in the laboratory (either with engineered cells or with a computer simulation), and so if we wish to understand the capacity and characteristics of this system we ultimately have to work with animals. We can undertake some experiments in humans, but the level of control over experimental conditions (e.g. long-term patterns of light exposure) and the range of techniques suitable for measuring and manipulating brain activity is far smaller in humans than laboratory animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our long experience of undertaking experiments of this type to decide the

best approaches to address our objectives and the minimum number of animals required to acheive those goals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Wherever possible we will compare a single animal's response to different conditions rather than use two different animals. This more than halves the number of animals used because it reduces the impact of inter-individual variation. In our electrophysiological experiments we use the latest equipment that allows us to record the activity of large numbers of neurones simultaneously from a single individual, greatly reducing the number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will routinely make use of tissue collected from animals at the end of experiments for analysis in the laboratory. We also undertake advanced statistical analysis and modeling of these data so that we can refine our questions and employ the most informative experiments. We also provide our data to other groups so that they can analyse it to answer their own questions. We will minimse the number of animals bred by using efficient breeding strategies ourselves and obtaining animals from commercial breeders 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.

We will be using laboratory mice and four-striped mice (*Rhabdomys pumillio*). Both of these species are accustomed to laboratory conditions. We use laboratory mice because we are able to build upon a wealth of existing information about the visual and circadian systems in this species, and because we have access to animals carrying naturally occurring mutations or engineered genetic modifications that are very useful for our objectives. Mice also allow us to study how vision works under dim light. A limitation of laboratory mice is that their visual system is adapted for dim light vision. Traditionally, this has led researchers to employ primate or companion animal species (especially cats) in vision research. We have established the four striped mouse as a rodent alternative which has good daytime vision. Working with four-striped mice allows us to understand how vision works under daytime conditions.

Why can’t you use animals that are less sentient?

Although non-mammalian vertebrates have retinas that are rather similar to those of humans, their visual systems differ from our own in other key respects. Most importantly,

unlike mammals, they have a wide variety of light sensitive cells outside of the retina. For this reason we have no alternative but to work with mammals.

Rodent visual systems develop after birth, meaning that we cannot address our questions at a more immature life stage. We include work on tissue harvested from terminally anaesthetised animals whenever possible, but this cannot replace studies of the intact visual system or when we wish to understand how behaviour is controlled.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will apply pain killers to reduce welfare costs following surgery. We carefully monitor animals during the recovery period after any procedure capable of causing pain or suffering. Wherever possible we will undertake brain recordings from animals under terminal anaesthesia. We use advanced 3D pose analysis methods developed in the group to provide automated and objective analysis of mouse behaviour, maximising the amount of information available from each experiment and avoiding the potential for observer bias in scoring behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We consult protocols, training resources and guidelines on best practices in animal experiments available through the NC3Rs website (https://www.nc3rs.org.uk/3rs-resources) and will adhere to them whenever relevant. Topics covered include handling and restraint, euthanasia, humane endpoints, welfare assessment, anaesthesia, and analgesia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular 3Rs updates through the animal unit and our 3Rs Regional Program Manager.

163. Use of genetically modified biological assemblies to generate improved vaccines

Project duration

4 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccine, Infection, Virus, Immunogenicity

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 will apply genetic modification to proteins which spontaneously assemble to form cage-like structures. I aim to demonstrate that these assemblies can be used as ‘scaffolds’ to form the basis for development of novel candidate vaccines against different infectious diseases, including Covid-19, whipworm and gonorrhoea.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 infectious diseases for which effective vaccines have not been developed, or provide limited protection in vulnerable people (eg the elderly). Vaccines are a highly effective way of protecting the public and have been instrumental in reducing or eliminating many infectious diseases. New technologies offer the potential for the development of more effective vaccines against a wide range of different diseases, but

they require testing in animals to ensure their safety and efficacy before their use in human trials.

This proposal relates to a specific technology which we have developed relating to the use of protein assemblies: this general term refers to the use of protein subunits which assemble into spherical structures. Such assemblies can be used to display specific vaccine antigens: antigens are molecules which are able to invoke an immune response. Vaccines incorporate antigens from infectious organisms and use these to stimulate a protective response, often by stimulating the production of antibodies against them. Protein assemblies are particularly effective at doing this: they can therefore be thought of as 'scaffolds', in the sense that they provide a molecular superstructure to which particular antigens can be bound. It is well established that virus-like particles can be used as a type of scaffold- these are virus proteins which assemble into a viral particle or shell, but are not capable of causing disease because they lack most of the components necessary for infection. Such assemblies are known to have useful immunological properties which make them particularly effective as vaccines.

Our technology allows the coupling of multiple antigens to a molecular 'scaffold' or support, in such a way that vaccines can be formulated easily with multiple components. This is a generic method, in principle applicable to the development of a vaccine against any infectious disease. We expect it to be particularly valuable for vaccines which require multiple components, or the immune response needs to be modulated in a specific manner. We anticipate that it may be able to overcome challenges which have limited vaccine development against specific diseases, including poor immunogenicity or an inappropriate immune response.

What outputs do you think you will see at the end of this project?

I anticipate that I will be able to demonstrate appropriate immunogenic responses to antigens derived from several important human pathogens, including the Covid-19 virus, the bacterium *Neisseria gonorrhoeae* (responsible for gonorrhea) and the whipworm parasite.

Who or what will benefit from these outputs, and how?

The recent Covid-19 pandemic has highlighted the importance of developing new vaccine technologies, particularly to protect against diseases for which there are no effective treatments, or where there is escalating resistance (eg to antibiotics). This project aims to validate a generalised approach to this problem which could be applied, in principle, to any infectious disease. The impact could be wide-ranging but will be after the conclusion of the project: vaccine development is generally a slow process and it typically takes many years for a new product to reach the market. It is important to note, however, that vaccines have saved the lives of millions of people and animals worldwide. We urgently need new approaches to address those diseases which, for a variety of reasons, have been resistant to vaccine development up to this point. It is only through further research and innovation that we will be able to overcome these challenges.

How will you look to maximise the outputs of this work?

The results will be disseminated primarily through publication in reputable peer-reviewed scientific journals, to ensure that it is subject to the scrutiny of the scientific community. The work will be presented at international conferences, which provides an opportunity for

informal feedback and discussion. I will also seek to develop the work commercially: if appropriate, I will seek patent protection for any inventions which emerge from the project.

Species and numbers of animals expected to be used

- Mice: 650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Development of new vaccines requires measurement of their ability to generate protective immune responses. The principle of a vaccine is that it attempts to reproduce the protective response of the immune system to an infectious agent- a virus, bacterium or parasite. Orchestration of the immune system is complex, involving many different types of specialised cells distributed throughout the body. Although aspects of the immune response can be modelled *in vitro* to a limited extent, it is not currently possible to reproduce it in its entirety without studying responses in the whole animal. Mice are commonly used in vaccine studies, making our results easier to compare with those obtained by others. Adult animals are most suitable for these experiments.

Typically, what will be done to an animal used in your project?

Each mouse will be inoculated up to 3 times with a small quantity of an experimental vaccine. Each dose will be separated by about 10 days; at the end of the experiment, each animal will be humanely killed before collection of blood and body tissues for analysis. Animals will be routinely monitored for any unanticipated adverse effects.

What are the expected impacts and/or adverse effects for the animals during your project?

The impact of our experimental vaccines is expected to be minimal- they consist of non-infectious protein and are unlikely to cause any harm to the animals. To minimise the risk of this happening still further, our candidate vaccines are tested for toxicity before administration. Vaccines generally consist of low quantities of material and are designed to be safe; adverse reactions are rare.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that all the mice will experience only mild discomfort during the inoculation steps. Given the low dose and minimal toxicity of the administered vaccine material, I anticipate that the majority of inoculated animals will experience no or minimal reaction. It is possible that a minority of inoculated animals may experience a reaction; for this reason, the maximum severity expected is designated 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?

It is not possible to reproduce the highly complex immune response induced by a vaccine without experimentation on the whole animal. Vaccines induce immune responses which require the coordination and interaction of multiple different cell types: B-cells, T-cells, dendritic cells and others. This complex network of interactions had not been modelled to a level of precision which can substitute for experimentation on the whole animal. Moreover, it is critical that we verify our vaccines are safe, as well as effective, before testing them in humans.

Which non-animal alternatives did you consider for use in this project?

The following non-animal alternatives are already in use as part of the project, to optimise the vaccines before testing on mice. I use computational methods to select the most promising protein fragments to use, and eliminate any which are predicted to cause adverse reactions. I also use dendritic cells to test for vaccine uptake and identify whether any of our candidate vaccines are cytotoxic (can kill cells).
Dendritic cells are cells which play an important part in processing antigens and directing the immune response. Our vaccine samples are also tested for any contamination which might cause an adverse inflammatory response.

Why were they not suitable?

The alternatives are valuable in refining the vaccine compositions but they only test very limited aspects of the immunogenic response induced by each sample. An immunogenic response is dependent on many different factors- the nature of the protein(s) used, the dose, the manner and timing of administration and others. It is currently not possible to predict the amplitude and type of immunogenic response which a vaccine will induce but it is vital to have this information before moving to human trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimate is based on our previous experience, and published details from others, who have administered similar vaccines to mice in the past. We have modelled the effects

which we anticipate and have based our estimate of total numbers on those statistical predictions.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have used statistical modelling, including the NC3R's Experimental Design Assistant, to model the effects which I anticipate for the vaccines I propose to use. Our experimental design compares responses between groups of animals who are administered the same vaccine and makes pairwise comparisons to derive statistically valid conclusions about the effects of particular parameters (eg the effect of certain antigens, dosage level, inclusion of adjuvant). Using these estimates, I have devised an experimental strategy which allows us to derive the maximum amount of necessary information for the minimum number of animals tested.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As detailed in previous sections, we have used computational methods and studies on isolated cells to screen our vaccines before administering them to animals. Computational methods allow us to identify which parts of each specific protein antigen are most likely to elicit an immunogenic response; such methods are not infallible, but they provide a useful basis from which to refine our vaccine composition. In addition, we make optimal use of the tissue from each animal, making multiple measurements from each individual. We can incorporate animals used from pilot studies into experimental groups, thus reducing the total number of animals used. Finally, we will use an iterative approach, through cycles of experiments, to identify the most important parameters and optimise them as efficiently as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

I will use the mouse as a vaccination model. The minimal requirement for such an experiment is the administration of vaccine, in up to three doses, through a suitable route. Each animal will therefore only be subjected to a maximum of three inoculations. Animals will be handled in such a manner as to minimise stress during inoculation. As detailed above, I will make extensive efforts to ensure that the administered vaccine material is not harmful or likely to trigger adverse responses. Safety and the avoidance of unwanted side-effects is an extremely important aspect of vaccine design. All samples will be tested on a smaller group of animals first, to verify no unanticipated adverse reactions. All animals will be monitored in the 24 hour period after inoculation for weight loss and any signs of distress or discomfort.

Why can’t you use animals that are less sentient?

The mouse is well established model for vaccine studies. Less sentient species have immune systems which are too different from humans, so the results provide a poorer indication of the likely responses in a clinical trial. In addition, there is a large body of published evidence which enables a direct comparison of the effects of particular vaccines in mice and humans. By doing our trials in mice, I will be able to compare our data directly with those findings and use it to extrapolate to the likely responses and safety in human subjects. I cannot use terminally anaesthetised animals because it takes weeks for immune responses to develop and it is not feasible to keep animals under anaesthetic for that long.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

I will conduct the minimum of experimentation necessary to collect the data which I require to exemplify the invention associated with this project. The work will proceed in experimental cycles, several months apart, which will allow me to learn and adjust the parameters of each experiment as I proceed. I will therefore refine critical parameters such as antigen selection, dose level and dosing strategy in an incremental manner, optimising immunogenic responses and eliminating any adverse effects, if observed. Refinement of the procedures, including optimised mouse handling techniques, is therefore an intrinsic part of our experimental approach.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to NC3Rs, technical advice and guidance is available to licence holders from the government (https://www.gov.uk/guidance/animal-research-technical-advice). Another valuable source of information is from regulatory bodies; for example, the Federal Drug Administration (FDA) has recently produced guidance for developers of COVID-19 vaccines, with specific advice on animal models (https://www.fda.gov/media/139638/download). Finally, I will continually survey the academic, peer-reviewed literature for publications which describe animal vaccine experiments which are similar to our own, to avoid duplication and allow us to refine our protocol further.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3Rs produces a newsletter and is a valuable hub for dissemination of good practice in animal experimentation. Staff engaged in the project will, in addition to mandatory training, be encouraged to engage with webinars from NC3Rs and elsewhere which provide up-to-date advice and information on best practice.

164. Biomechanics and signalling in the developing cardiovascular system

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cardiovascular development, Valve formation, Biomechanics, Mechanotransduction, Mechanical forces

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project addresses the role mechanical forces during the formation of the cardiovascular system. It aims at elucidating how cardiac cells modulate gene activation in response to physical stimuli generated by blood flow during normal heart valve development and regeneration in zebrafish. This knowledge will result in a better understanding of the heart function and 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?

Cardiovascular diseases (CVDs) take a huge toll on the world population. An estimated 17.9 million people died from CVDs in 2016, representing 31% of all global deaths. Heart valve defects are the leading cause of congenital cardiac malformation in human and originate during embryonic development. Cardiac valve formation requires a complex set of cellular rearrangements and is significantly affected by the physical forces generated by blood flow. Blood circulation is critical for valve development. It generates a variety of

mechanical forces that are sensed by cells and activates cell response, a process termed mechanotransduction. Abnormal blood circulation is widely recognised as a cardiovascular risk factor and mechanotransduction has been shown to trigger pathologies such as atherosclerosis and pathologies of the heart valves (valvulopathies). However, the mechanism in which physical stimuli control genetic pathway activation and valve development remains elusive.

This project intends to decipher the cellular and molecular programmes activated in EdCs in response to mechanical forces during normal heart valve development and examine their contribution in valve regeneration, using zebrafish as a model organism. This knowledge will provide new insights into the heart's activity and comprehensive analyses of cardiac dynamics, resulting in a better understanding of the heart function and development which could pave the way for future medical advances.

What outputs do you think you will see at the end of this project?

An estimated 19 million people died from cardiovascular diseases (CVDs) in 2010, representing 30% of all global deaths. Abnormal blood circulation is widely recognized as a cardiovascular risk factor and abnormal mechanical forces can trigger pathologies such as atherosclerosis, cardiac disease and cardiac valve defects. Mechanical stimuli can be converted into biological responses. It is central to the coordination between mechanical forces generated by flowing blood and heart formation. Our project places mechanical forces in the focus of effectors of cardiac development. It will decipher how cardiac cells modulate gene activation in response to physical stimuli generated by blood flow during normal heart valve development and regeneration in zebrafish. This knowledge will result in a better understanding of the heart function and development which could pave the way for future medical advances.

In the context of this research, we will develop imaging tools, genetic lines and produce gene expression data that shall be of considerable interest to other researchers in the field of heart and other organs development and regeneration. Sharing such tools and knowledge will help scientific advances be achieved in shorter periods of time and will minimise experiments using animals by avoiding repetition.

Who or what will benefit from these outputs, and how?

Short-term benefits

Our scientific project will be highly instructive to early stage researchers and will further train advanced researchers. PhD students and Postdoctoral fellows recruited within this proposal will profit from an international and interdisciplinary environment, combining molecular biology, developmental biology, bioinformatics, physics and engineering. Their projects will involve the use of multiple techniques, address relevant biological questions and shall therefore allow them to develop as researchers, embracing the value of effective training.

Medium-term benefits

The project will contribute technological developments for in vivo imaging of the endocardial cell response to mechanical stress; a key quantitative correlation which is almost impossible to implement in other species. Cardiac regeneration is not exclusive to the zebrafish, but has also been observed in newborn mice and stem cell tissue

engineering is now emerging as a key technology for the future of valve replacement. Therefore, lessons learned from studies in the zebrafish will be possible to translate to mammalian systems. The tools, including imaging tools, genetic lines and gene expression data, that we will develop will be also of considerable interest to other researchers in the field of heart and other organs development and regeneration. Sharing such tools and knowledge will help scientific advances be achieved in shorter periods of time and will minimise experiments using animals by avoiding repetition.

Long-term benefits

This project focuses on the factors that control cardiac development activated in response to mechanical forces. It will decipher how cardiac cells modulate gene activation in response to physical stimuli generated by blood flow during normal heart valve development and regeneration in zebrafish. Thus, patients could be indirectly benefiting from these findings in the future when it comes to treat cardiac valve defects.

How will you look to maximise the outputs of this work?

To make our results available to the scientific community as soon as possible we will communicate our work through the different social media channels and will pursue the publication of our results in online repositories, as well as send them to peer-reviewed well recognized journals in the field of developmental biology, cardiovascular biology or molecular and cell biology. We will also participate in cardiovascular and zebrafish conferences to present our work.

Our proposal will be highly instructive to early stage researchers and will further train advanced researchers. PhD and Postdoctoral fellows recruited within this project will profit from an international and interdisciplinary environment, combining molecular biology, developmental biology, bioinformatics, physics and engineering. Their projects will involve the use of several techniques and will answer relevant biological questions, allowing them to grow as researchers.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 31000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 (ZF) is the model organism we will use and it is one of the most widely used animal models for developmental and regeneration studies. Adults give rise to large numbers of embryos, which can be maintained completely translucent for up to 5 days post-fertilisation (by which time organogenesis has been completed), providing a significant advantage for heart imaging. In addition, TALEN and CRISPR-mediated genome engineering are possible. Together with novel optical technologies, which our lab has developed to study mechanical forces, these features provide exclusive tools to study valve development and physiology in response to mechanical stress.

The life stages of interest include larvae older than 5 days post-fertilisation and juveniles younger than 89 days post-fertilisation. These stages are selected based on specific developmental stages of the heart, as the valves continue to mature at juvenile stage. Key morphogenetic processes are occuring after day 5 consisting in valve leaflet growth and proliferation of important valve cells. We thus need to assess valve morphology and valve cells at later stages as well.

Typically, what will be done to an animal used in your project?

Eggs or sperm will be obtained (by applying gentle pressure on/or stroking the sides of the fish) for experimental use in vitro fertilization or germplasm freezing. Generation of founders (by direct zygotic injections and in vitro manipulation of gametes or zygotes, blastulae, embryos and/or fertilisation) and production of genetically altered (GA) zebrafish will follow. Progeny may be processed for gentle imaging using live microscopy, e.g. light-sheet microscopy. For the imaging purposes, animals will be anaesthetised (anaesthesia will never exceed 15 minutes) and mounted in low melting-point agarose. At the end of the imaging process animals will be killed according to a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

While maintaining the population, some fish may have the potential to develop a harmful phenotype after a certain age, but in all cases will be killed before reaching that age and before onset of clinical signs, unless moved on to another protocol as continued use for a specific purpose. Fish exhibiting any unexpected harmful phenotypes will be killed by a schedule 1 method, or in the case of individual fish of particular scientific interest, advice will be sought promptly from the assigned Home Office Inspector. Fish will be immediately killed by a schedule 1 method if they show signs of suffering that is greater than minor and transient or in any way compromises their health or wellbeing (for example fish that do not grow, behave, swim and feed normally.)

Type and depth of anaesthesia will be carefully selected and monitored in consultation with the NVS. Fish that do not return to normal swimming behaviour within 30 minutes after removal of the anaesthetic will be killed by a schedule 1 method.

During gamete collection, massaging the abdomen of fish could cause scale loss leading to a breach in the epidermis and/ or dermis leave the fish vulnerable to infection (<1%) or could cause compression damage to internal organs (< 1%). Gametes should be released readily; therefore, no attempt should be made to force gamete release. Any fish that develop infection or exhibit any abnormal behaviour on recovery from anaesthesia will be killed by a schedule 1 method.

Genotyping: The site and amount of tissue removal will be such that there is no compromise to normal swimming. Following fin clipping for genotyping in >5dpf fish peri-operative analgesia will be provided; agents will be administered as agreed in advance with the NVS. Any fish exhibiting any abnormal behaviour will be killed by a schedule 1 method.

Infections can result from fin clipping (<1%) or from damage to scales or loss of mucous surface from swabbing. The procedure will be carried out using sterile equipment. Fish that develop signs associated with infection will be killed by a schedule 1 method.

Injected constructs may cause death or developmental abnormality before fish reach the stage of protection. Harmful genetic alterations in embryos may be evident as altered morphology prior to hatching. Embryos will be assessed for morphological phenotypes before the stage of independent feeding and any showing morphological abnormality not required for the scientific purpose would be killed using a schedule 1 method. Some genetic alterations may result in a harmful phenotype during post-hatching development evidenced as failure of larvae to inflate the swim bladder, difficulty swimming, altered morphology, failure to feed or breathing difficulties. If fish exhibit any of these adverse effects they will be killed immediately by a schedule 1 method. On occasion (<5%) late onset mutations in more mature stages may lead to lines of fish with mild lordosis (defined as less than 20% curvature of the spine), poor growth /body condition or difficulty swimming. Any fish showing harmful phenotypes will be killed by a schedule 1 method as soon as the phenotype is visible.

in vivo imaging of anaesthetised zebrafish in larval and juvenile stages: fish may exhibit abnormal behaviours or show signs of suffering that is greater than minor and transient or in any way compromises their health or wellbeing as a result of anaesthesia.In this case, animals will be immediately killed by a Schedule 1 method. Type and depth of anaesthesia will be carefully selected and monitored as advised by the NVS and anaesthesia will last less than 15 minutes. Fast, high- resolution live heart imaging will be performed, e.g. light-sheet microscopy, yet adverse effects are not anticipated during the imaging experiments as these methods have been previously proven safe for the imaging of the sensitive developing animals. In case of adverse effects encountered, the animals will be humanly killed in accordance to Schedule 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)?

Mild severity is expected in all 4 Protocols. All animals will experience mild severity.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Up to the present moment, there are no alternatives to the use of animals for the study of vertebrates early development. Even though invertebrates have been an important source of information for the basic biology of embryonic development, they cannot replicate the complicated nature of the development of vertebrates. Computer models and in vitro approaches fail to do so, too.

Furthermore, the cardiovascular system in zebrafish is made of cells with specific mechanical properties which are not present in invertebrate models and cannot be replaced by computer models. Our project is directed towards addressing how the cells populating the cardiovascular system integrate mechanical information to form and maintain a functional cardiovascular system. It is essential to determine how mechanical forces control gene activation and cardiovascular development in vivo since the mechanical stimuli experienced by cells of the cardiovascular system are too complex to be faithfully reproduced in vitro or in silico.

Which non-animal alternatives did you consider for use in this project?

We considered developing and using approaches allowing to study the effects of mechanical forces on cultured endothelial cells. Yet, in vivo approaches are still essential to study the system in its native environment since the mechanical forces generated are still too complex to recapitulate in vitro.

We also considered using computer models, however, up to the present moment they fail to replicate the complicated nature of the development of vertebrates.

Why were they not suitable?

Suffice alternatives have not been developed yet.

Computer models and in vitro approaches fail to replicate the complicated nature of the development of vertebrates.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 taking into account the experimental procedures that will be conducted and by implementing appropriate statistics, we cautiously designed experiments that will ensure the minimum number of animals required to answer scientific questions robustly.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to minimise the number of animals used, we will only generate new transgenic lines if they are not already available. Sharing data and resources with other research groups and organisations can 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?

In order to minimise the number of animals used, we will only generate new transgenic

lines if they are not already available. Sharing data and resources with other research groups and organisations can also contribute to reduction. By implementing appropriate biostatistics, we will determine the minimum number of animals that will be utilised during the project in order to achieve statistical power with the obtained results. Moreover, we will strive to keep our husbandry conditions as stable as possible using standardised food regimens, monitoring water quality regularly and using the same rearing conditions and our experiments will be rigorously planned to avoid major pitfalls and thus repetition of protocols.

We also plan to develop and use approaches using cultured endothelial cells and computer models allowing to study the effects of mechanical forces. Yet, in vivo approaches are still essential to study the system in its native environment since the mechanical forces generated are still too complex to recapitulate in vitro or in silico.

All of the experiments included in this licence will be planned so they can be published in accordance with the NC3Rs ARRIVE guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish (ZF) is the model organism we will use and it is one of the most widely used animal models for developmental and regeneration studies. Adults give rise to large numbers of embryos, which can be maintained completely translucent for up to 5 days post-fertilisation (by which time organogenesis has been completed), providing a significant advantage for heart imaging. In addition, TALEN and CRISPR-mediated genome engineering are possible. Together with novel optical technologies, which our lab has developed to study mechanical forces, these features provide exclusive tools to study valve development and physiology in response to mechanical stress.

The methods we will apply include:

breeding and maintenance of genetically altered zebrafish, where fin clips and swabs will only be taken if more refined genotyping methods are not suitable scientifically

obtaining zebrafish gametes, which is the most efficient method for large-scale and long-term storage of important genetic material. Massaging the abdomen of fish could cause scale loss leading to a breach in the epidermis and/ or dermis leave the fish vulnerable to infection (<1%) or could cause compression damage to internal organs (< 1%). Gametes should be released readily; therefore, no attempt should be made to force gamete release. Fish will always undergo at least one recovery gamete collection before a collection under terminal anaesthesia

generation of founders, where GA embryos will be generated by in vitro manipulation of

gametes or zygotes, blastulae, embryos and/or fertilisation. Viable embryos will be raised to generate founders. Harmful genetic alterations in embryos may be evident as altered morphology prior to hatching.
Embryos will be assessed for morphological phenotypes before the stage of independent feeding and any showing morphological abnormality not required for the scientific purpose would be killed using a schedule 1 method. Some genetic alterations may result in a harmful phenotype during post-hatching development evidenced as failure of larvae to inflate the swim bladder, difficulty swimming, altered morphology, failure to feed or breathing difficulties. If fish exhibit any of these adverse effects they will be killed immediately by a schedule 1 method

in vivo imaging of anaesthetised zebrafish in larval and juvenile stages. Type and depth of anaesthesia will be carefully selected and monitored as advised by the NVS. Fast, high-resolution live heart imaging will be performed, e.g. light-sheet microscopy, yet adverse effects are not anticipated during the imaging experiments as these methods have been previously proven safe for the imaging of the sensitive developing animals. In case of adverse effects encountered, the animals will be humanly killed.

Why can’t you use animals that are less sentient?

Until now, there are no alternatives to the use of animals for the study of vertebrates early development. Even though invertebrates have been an important source of information for the basic biology of embryonic development, they cannot replicate the complicated nature of the development of vertebrates. Furthermore, the cardiovascular system in zebrafish is made of cells with very specific mechanical properties which are not present in invertebrate models.

The life stages are selected based on specific developmental stages of the heart, as the heart valve matures at larval and juvenile stages. These stages are particularly relevant for the understanding of pathological valves as valves often display pathological signs (valve distrophy or hypertrophy) at these stages. Without assessing these late stages, we may miss important valve anomalies associated with abnormal mechanical forces.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our animals will be housed under pathogen free and environmentally controlled conditions that allow the expression of species-specific behaviours. NVS advice will be followed to maintain the animals in a healthy condition/ free from opportunistic infection. Procedures that can possibly cause pain, suffering or distress will be performed under anaesthesia as indicated to minimise these effects and animals experiencing traumata or severe adverse effects that cannot be relieved will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the latest guidance published by the NC3Rs and follow the latest ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are several ways to keep abreast of the latest 3Rs developments. Having regular discussions with the Named Persons and animal technicians at our establishment to review current approaches and whether there are any new 3Rs opportunities, subscribing to the NC3Rs e-newsletter, attending NC3Rs events, workshops and Institutional 3Rs symposia are simple ways of staying informed of 3Rs advances and approaches. Furthermore, having access to a local NC3Rs Regional Programme Manager and maintaining contact with them can provide an informal route to 3Rs advice, developments and best practice.

165. Circuit mechanisms of learning and memory in the mammalian brain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

brain, learning and memory, synaptic plasticity, circuit mechanisms, behaviour

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 understand how brain activity leads to changes in connections between cells, and how these changes in connections can explain memory.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how we learn and remember has fascinated scientists for hundreds of years. It is believed that changes in the connections between cells in the brain underpin learning and memory. However, how these changes affect behaviour is poorly understood. This work aims to identify how cell activity induces changes in the connections between cells. Conversely, we will identify how these changes in connections affect activity. Learning and memory are fundamental properties of the brain.
They enable animals to adapt to their environments. Memory is also essential for our

identities as humans. Human brain disorders that affect memory have devastating effects on the individual as well as the society. As such, insight into memory processes is important not only for understanding animal behaviour but also for the treatment of learning disabilities and memory disorders in humans.

What outputs do you think you will see at the end of this project?

This project is aimed at generating new knowledge. Whereas we know much in theory about how connections between brain cells can change, what activity controls these changes in the intact brain during learning and memory is not known. The mechanisms involved and what goes wrong in human learning disabilities and memory disorders are also poorly understood. In this project, we expect to confirm whether or not the theory for how changes in connections occur actually operates in the intact brain, and whether they are responsible for behavioural learning and memory. We also expect to elucidate the fundamental mechanisms involved. Thus, the primary benefit of this programme of work is knowledge generation. The new knowledge would be shared with other investigators through publications in scientific journals, and the general principles would be shared with other people with an interest in memory and memory disorder through public lectures.

Who or what will benefit from these outputs, and how?

The short-term benefit of this programme of work is knowledge generation. This will inform other researchers as well as students. A longer-term benefit is better understanding of how defects in learning and memory mechanisms lead to disease. In particular, we will study developmental brain disorders and neurodegenerative diseases. The new insights gained could lead to new strategies for treating such disorders, including learning disabilities and Alzheimer's disease. However, this is not likely to be realised until after the project has been completed.

How will you look to maximise the outputs of this work?

We will aim to maximise the outputs of the work by collaborating with other scientists, including investigators working on human brain disorders in the clinic. We will also work with computational neuroscientists, who could help interpret the experimental results.

Species and numbers of animals expected to be used

- Mice: 27,750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen the mouse as the experimental species for three main reasons. First, mice are quite close to humans, evolutionary speaking. This makes our research findings relevant to mechanisms in the human brain. Second, mice are widely used to study brain mechanisms of learning and memory. Thus, we can build on results obtained by other investigators. Third, there are techniques available that can modify genes in mice. This genetic technology is powerful in addressing our scientific questions.

We also want to find out what goes wrong with learning and memory in developmental disorders of the brain. Therefore, we need to do experiments at all life stages, from embryos through newborns to adolescent and adult mice.

Typically, what will be done to an animal used in your project?

The majority of animals will be used for breeding of genetically altered mice. A relatively high number is necessary because several generations of mice are often required to generate the mouse lines to be used experimentally. During breeding the animals would express their natural behaviour. Some of these animals may show mild signs of brain disease, such as problems with learning and memory. Very few animals will show other problems, such as problems with movement in a mouse model of motor neuron disease with dementia.

Some of our experiments will be done on isolated brain tissue from mice that have been humanely killed.

A smaller number of experiments will involve brain surgery on animals that have been anaesthetised. This is to introduce genes locally in the brain; to make tiny brain injuries; to insert electrodes, light fibres, or tubes to deliver drugs into the brain; or to mount a bracket, which would later be used to keep its head still. At most two such surgeries would be made in an animal. Afterwards, we would prepare brain tissue or record activity in the brain during awake behaviour or sleep. Sometimes we would record while the animal performs simple memory tasks for food or water reward, after the animal has been made slightly hungry or thirsty. The memory tasks may take up to three weeks, with testing every day.

We will always strive to use the least harmful methods to achieve the results required to answer our scientific questions.

What are the expected impacts and/or adverse effects for the animals during your project?

Most animals will not experience any adverse effects.

Some animals serve as models of neurodevelopmental and neurodegenerative disorders. They will show mild behavioural changes, such as learning deficits. Any mouse that shows more than mild motor impairment will be humanely killed. Any animal will be immediately humanely killed if it shows signs of suffering that is likely to exceed those detailed for the animal model.

Some animals will undergo a surgical procedure.
Some animals will be food restricted to achieve 80% of free-feeding weight for a period of up to 28 days and/or experience short periods up to 4 hours of water restriction before behavioural tests. They would take place no more than twice per day for up to 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 severity limits of the protocols in this project are Non-recovery, Mild and Moderate, and the proportion of animals in each category is:

Mice: Non-recovery - 20% Mild - 65%
Moderate - 15%

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 use of animals is necessary in this project, as there are no other experimental models that can elucidate brain mechanisms of memory.

Which non-animal alternatives did you consider for use in this project?

We will complement our studies using human neurons. This may better reflect mechanisms of human memory disorder.

We will also complement our animal studies with models on computer to better understand how our discoveries can explain memory.

Why were they not suitable?

These non-animal alternatives can complement animal experiments but can not directly investigate the circuit mechanisms of learning and memory in the mammalian 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 estimate is based on previous experience of breeding and animal experiments. The majority of the estimated number is related to the breeding programme of genetically altered animals (17,000). From these animals we will be collecting tissues for use in experiments in the laboratory, thus reducing the need for experiments in live animals. The remainder would be experiments under anaesthesia for collection of brain tissue. At most 4,250 surgeries would be made in which the animals would wake up again following

surgery.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our research is fundamental discovery science. As such the research proceeds in three stages. First, we notice an unexpected result in our data, which gives us new insight and ideas about learning and memory processes. Second, we articulate and develop those ideas to generate a research question. Third, we verify our insight by addressing the research question in experiments. It is very difficult to know in advance exactly how many animals would be needed to address our research questions, but statistics will help us to balance the number of animals used and the confidence we can have that the answers we obtain are correct.

Using statistics, it is possible to gauge in advance how many animals would be required to answer a specific research question. This is important in order to know how likely it is that other investigators would reach the same conclusion if they carried out the same or similar experiment. Typically, we would need at least 20 animals in each of two groups to be reasonably safe to detect a difference between the two groups.

The most important measure we use to reduce the number of experiments conducted on live animals, however, is to do experiments in brain tissue collected from animals after they have been humanely killed. This is then used to understand the mechanisms for the phenomena we observe in intact animals during behavioural learning and memory.

We will consider using NC3R's Experimental Design Assistant (https://www.nc3rs.org.uk/experimental- design-assistant-eda) when appropriate for our behavioural experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To reduce the number of animals in the breeding programme:

Efficient breeding: We will use a breeding programme that maximises offspring with the required genes.

Use of spare animals: We will use animals without the required genes in other experiments, for example to prepare brain tissue.

Collaboration: We will share the genetically altered animals with other laboratories.

To reduce the number of animals used in experiments:

Pilot studies: We will do studies with a small number of animals in order to plan a more conclusive study.

Computer modelling: We will use simulations on computer to better understand our experimental observations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 to study mechanisms of learning and memory in the brain. In order to record and control activity in the brain, we will use a technique called 'optogenetics'. This technique allows us to label specific cell types, record their activity using miniature microscopes, and stimulate or silence them while an animal learns a behavioural task. This technique will also enable us to target the same cell types in brain tissue taken from the animal after it has been humanely killed and nerve cells grown in cell culture.

Some animals will be genetically altered to model human brain disease. Almost all of these animals will show only mild signs of disease. A small number might develop problems with movement in a mouse model of motor neuron disease with dementia, but will then be immediately killed.

Why can’t you use animals that are less sentient?

We will use cell culture prepared from baby mice and brain tissue from mice of any age to study mechanisms. However, mechanisms involved in natural learning and memory can only be studied in awake behaving animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will monitor animals carefully in all breeding procedures. For animals with progressive disease, we will only use young breeders, and replace breeders if they show signs of disease, for example muscle weakness in motor neuron disease.

We will follow the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2nd edition, 2017) for all surgical procedures, and ensure appropriate pain management. To assist with animal welfare, we will use monitoring and observations charts to follow the recovery of the animal. Recovery is expected to be uneventful, but the monitoring would enable us to humanely kill the animal if the animal is suffering.

The investigators who study mouse behaviour will be trained for this purpose and will use rewards, rather than punishments, to encourage learning in behavioural tasks. All animals will be gradually acclimatised to the behavioural apparatus. Some animals will have a small bracket attached to their head. This will be used to restrain their heads in some of the experiments while mounting a recording device.

To reduce the inescapable harms arising from using animals in research, we have considered the following points:

To reduce the transportation of animals, we will import the minimum number of breeding pairs and continue a breeding programme in house. We will consider importing frozen

embryos instead of adult animals when possible.

To the extent possible, we will co-house animals. In rare cases, we have to single-house animals because of sensitive implantation devices, but even in those cases, we will try to keep them at least in pairs.

We will improve the husbandry of mice in behavioural experiments by using enrichment cages where mice have access to two floors, running wheels, and cardboard houses and tunnels and where they can be housed up to ten in each cage.

Amendments March 2022: To reduce animal stress when positioning a mini-camera on the animal's head for the first time, we will in some animals do this under brief and light general anaesthesia.

Moreover, to minimise the welfare costs for the animals, instead of using light to control the activity of neurons in the brain, which requires implantation of a light guide into the brain, we will in some experiments inject an inert chemical substance acting on genetically inserted receptors. This enables control of neural activity without the need for surgical implantation of a light guide.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In planning experiments, we will consider the PREPARE Guidelines (Smith et al., Laboratory Animals, 2017) and the 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.). We will consult position papers from LASA (https://www.lasa.co.uk/current_publications/). Specifically, for surgeries, we will follow the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2nd edition, 2017). We will be guided by the updated ARRIVE Guidelines 2.0 (Percie du Sert et al., PLoS Biol 2020) when reporting results from our research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will seek information at the web pages of NC3Rs (https://www.nc3rs.org.uk), LASA (https://www.lasa.co.uk), and Norecopa (https://norecopa.no). Moreover, our institution's web pages provide regular updates on new developments in the 3Rs. We will consider these advances carefully to see whether they can be implemented effectively in this project.

166. Oxygen sensing and nutrient signalling in metabolic disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

obesity, diabetes, metabolism, mitochondria, hypoxia

Animal types	Life stages
Mice	neonate, juvenile, adult, embryo, pregnant
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To study how oxygen levels and nutrients, particularly lipids, are sensed in tissues during metabolic diseases, and how this contributes to the pathology.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 chronic metabolic disease continues to rise in the developed world, associated with the rise in obesity levels and associated conditions often termed lifestyle diseases. Many such conditions (e.g. type 2 diabetes, cardiovascular disease, fatty liver disease) worsen over time and whilst lifestyle changes can slow the progression, therapeutic options are very limited at present. It is vital, therefore, that we develop a greater understanding of the biological mechanisms underlying these conditions and the

high mortality rates associated with them. In so doing, we can understand why mortality is so unacceptably high, as well as identifying potential targets for therapeutic intervention that could positively impact on patient health, improving survival and quality of life.

What outputs do you think you will see at the end of this project?

The direct academic benefits of this work include:

A better understanding of the molecular signalling pathways at play in metabolic diseases such as obesity, diabetes and fatty liver disease. Chiefly, we are interested in developing new understanding of nutrient signalling (e.g. how fat molecules, such as those found in the diet, can regulate cell function) and oxygen sensing. In particular, we will seek to establish whether molecules involved in oxygen sensing pathways are activated in metabolic disease, and if these play a key (causative) role in the disease progression.

An improved understanding of the alterations in the lipid composition of tissues in metabolic disease. Lipids are a diverse class of hydrocarbon molecules that includes fat molecules and sterols such as cholesterol. Lipids have a wide range of functions and play roles in the cell including as fuel for energy metabolism in the mitochondria (the powerhouses of the cell), but also as signals that can alter cell function. Lipid composition is altered in metabolic disease, but we aim to indicate which lipids play a key (causative) role in the disease progression. In particular we wish to focus on how lipids alter the capacity of the cell (and the mitochondria within the cell) to convert chemical energy in molecules from the diet into a usable form of energy that can support cell, tissue and organ function to sustain life.

An indication of possible therapeutic targets (identified through the use of genetically-altered animals) and/or therapeutic strategies through the use of agents that modify lipid/oxygen sensing.

The research in this project will bring together information using a wide range of biological techniques to study the concentrations of important molecules alongside different aspects of cell, tissue and organ function, in conjunction with measurements made on living animals. For researchers working in disciplines related to ours, this comprehensive analysis will be of significant interest and provide a great resource for the analysis of associations between different sets of data, leading to the generation of new hypotheses.

The specific outputs will include new information in the form of data, resulting in publications in peer- reviewed scientific journals including primary research articles, review articles and commentary pieces. We will aim to make the supporting data underpinning these publications available whenever possible. We will share full datasets with collaborators when appropriate and more widely after we have published our own findings.

We do not anticipate generating any new products, but we will work closely with our current industry collaborators throughout the project, using existing compounds to modify metabolism and signalling pathways. As such, our work may highlight new uses for existing compounds or indicate new targets for future compounds.

Who or what will benefit from these outputs, and how?

The beneficiaries of this research, in the short to medium term (as publications arise) will include:

metabolic physiologists, particularly those with an interest in the factors that control lipid metabolism, in specific tissues and systemically, in response to altered conditions in the cell;

mitochondrial biologists, especially those with an interest in the role that mitochondria play in the hypoxia response, which includes oxygen sensing and signalling aspects, through the generation of reactive oxygen species, and the effect of hypoxia on mitochondrial respiratory function and substrate metabolism;

researchers interested in the molecular pathways underpinning the physiological response to hypoxia, including the hypoxia-inducible factors and their interaction with a class of free radicals, known as reactive oxygen species;

In the longer-term, the implications of these findings will become more fully understood, leading to further research into applications and here beneficiaries may include:

clinicians and clinical researchers with an interest in the aetiology of metabolic disease, where mitochondrial dysfunction secondary to hypoxia and oxidative stress has been implicated as playing a key role;

researchers in the pharmaceutical industry looking to identify new possible therapeutic targets or therapeutic approaches for the treatment of metabolic disease;

researchers in universities and the pharmaceutical industry with an interest in toxicology and medicines safety, since hepatic hypoxia is an underlying feature of many pathologies and is likely to alter the capacity of the liver to metabolise pharmaceutical agents, particularly those with a known effect on mitochondrial function.

The research in this project is highly integrative, including analyses of whole animal physiology alongside measures of mitochondrial respiratory function, reactive oxygen species production, morphology and tissue distribution, as well as comprehensive analysis of tissue and circulating lipid and non-lipid metabolites, free radical species and relevant transcriptional pathways. For researchers in all disciplines outlined above, the comprehensive analysis across a range of platforms will be of significant interest and provide a great resource for network analysis, leading to the generation of new hypotheses and grant proposals.

How will you look to maximise the outputs of this work?

We will disseminate the findings of our research via open access publications, and we enthusiastically support the concept of open data, so will make all underpinning research data available upon publication. In all publications, we will target journals to ensure that our findings reach the appropriate audience and ensure that our publications are open access. Moreover, we will work with the Establishment communications team to publicise our work. We will also present the work at relevant academic conferences and through invited seminars at other universities and research institutions in the UK and overseas.

Where possible, we will aim to publish unsuccessful approaches and negative findings, which we recognise are important to prevent duplication of procedures and reduce the number of animals used.

The collaborative and interdisciplinary nature of our work, which includes academic and industry partners, will ensure that maximum benefit is derived from this research and that the findings are communicated widely and to the most likely beneficiaries in a timely manner.

Species and numbers of animals expected to be used

- Mice: 500
- 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 are using weanling and sexually-mature adult mice and rats up to 12 months of age. These are the least sentient species suitable for studies of integrative metabolic physiology. Moreover, we can capitalise on established models of metabolic disease in both species.

We carefully consider whether rats or mice are appropriate for each particular study when designing our experiments. For instance, if we need to probe the role of a particular gene or pathway, there is greater availability of genetically-modified mice than rats. Nevertheless there are some rat strains which are of interest (e.g. the Zucker fatty rat, which is predisposed to develop obesity). It is sometimes necessary to use rats if larger quantities of tissue or blood are required than might be obtained from a mouse, and this will allow a greater number of measures to be made on a single animal, which both enhances the value of the data as a whole and reduces the total number of animals used.

Typically, what will be done to an animal used in your project?

We will be studying animal models of metabolic disease. These will typically be induced by feeding a modified diet (e.g. high-fat or high-fat/high-sugar diets). Animals may remain on these diets for up to 12 months but typically for a much shorter duration, and this would be expected to lead to altered metabolic function in tissues, obesity and possibly insulin resistance. In some cases, we will induce type 2 diabetes via the injection of a low dose of an antibiotic known to alter pancreatic insulin production. We will study some animals that have been genetically-modified in such a way that they are predisposed to develop metabolic disease or protected against the consequences of metabolic disease.

In addition, an animal may undergo one or more of the following procedures, but a typical animal will experience no more than five of these:

Animals may be exposed to low oxygen levels (hypoxia) in a chamber. In this case, animals will be exposed to hypoxia for less than one month and at levels that they are known to tolerate well.

Animals may receive drugs known to alter their metabolism, either through injections or orally. Not all animals will receive such drugs and the volume and number of injections used to administer drugs will be the minimum needed.

Animals may receive injections of molecules known as tracers which allow us to understand more about the metabolic processes taking place in their cells or the oxygen levels of their tissues. Not all animals will receive such substances, and no animal would receive more than 4 injections of tracers in their lifetime, and typically a single injection of tracer would be sufficient.

Animals may be housed alone for short periods of time (typically no more than 3-4 days, but on rare occasions up to 4 weeks). This would be necessary to measure the amount of oxygen they are consuming, their activity and food and water intake, or it may be necessary in order to match the food intake of a control animal to another animal in the study.

Mice may be scanned using nuclear magnetic resonance (NMR) to look at their body fat percentage. Animals are briefly restrained (less than 2 minutes) in a plastic tube. This does not involve anaesthetising the mice, but is painless and harmless.

Some animals will be anaesthetised for less than 1 hour so that we can measure the function of their hearts using an ultrasound probe. We need to anaesthetise the animals in order to obtain clear images of the heart without the animal moving around. No animal would be expected to undergo this more than 6 times in their lifetime with at least 1 week between each imaging procedure.

At the end of the experiment, each animal will be killed using the most humane method that is possible while still allowing us to collect blood and tissue samples that are not adversely affected by the method used to the detriment of the scientific analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals developing features of metabolic disease will show a range of adverse effects, which might include obesity and insulin resistance. They may become lethargic. They may urinate more. They may experience some general discomfort associated with fatigue, which might resemble premature ageing, but we would not expect them to experience pain. Some genetically-modified animals might be expected to show similar adverse effects, whilst others would be protected from the impact of metabolic disease and show less adverse effects.

Animals exposed to hypoxia experience some lethargy and increased ventilation rates, particularly in the first two days. Food intake may fall for the first two days (depending on the degree of hypoxia used). All animals would be expected to recover normal food intake and activity after this.

Animals would not be expected to experience any lasting harm from injections *per se*. Tracers would not be expected to cause any adverse effects. In this project, drugs which alter metabolism would typically be expected to lessen the adverse effects associated with metabolic disease, although occasionally this may slightly worsen the effect.

Single-housing can be stressful for mice and rats, but we will minimise the time each animal is housed alone and this causes no pain or lasting harm. Where possible, animals will be re-housed in groups with their original cage-mates following a period of single-housing.

Animals anaesthetised for cardiac ultrasound imaging will feel groggy as the anaesthetic wears off, but will experience no pain or lasting harm. The ultrasound itself is pain-free and harmless,

NMR scanning for body composition analysis causes no pain, stress, or lasting or immediate harm to 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)?

Most of the mice used in this project (> 75%) will experience sub-threshold or mild severities. A small proportion (< 25%) might experience a moderate severity.

Most of the rats used in this project (> 75%) will experience sub-threshold or mild severities. A small proportion (< 25%) might 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 project necessitates the use of animals in a study of integrative physiology, as we seek to investigate metabolic alterations in different tissue compartments and systemically (i.e. in the blood) as well as the interactions between these compartments. Moreover, we need to use validated models of metabolic disease in carefully-controlled studies. In order to mechanistically investigate the importance of particular genes on metabolic disease, in some cases in a tissue-specific manner, we need to use genetically-modified animals.

Which non-animal alternatives did you consider for use in this project?

We have considered cell culture models of altered metabolism, including different cell lines that reflect tissue-specific metabolic pathways. We have also considered the use of organoids e.g. liver organoids, which are miniaturised and simplified three-dimensional cultures of cells that, to some extent, reflect the function of an organ.

We have considered using clinical samples, including samples and data from large cohort studies.

Why were they not suitable?

There are currently no tissue or cell culture model alternatives to investigate the complex signalling networks associated with systemic/tissue hypoxia, mitochondrial function and lipid signalling, nor the interplay between different systems. Cultured heart muscle cells

(cardiomyocytes) do not experience the same workload or blood pressures as heart cells in the body. Moreover, liver metabolism is critical for whole-body metabolic control, playing vital roles in fuel storage and synthesis of lipids, steroids and ketone bodies and in detoxification. As such, the metabolic consequences of tissue hypoxia, alongside the administration of drugs that target tissue mitochondrial function, will extend beyond specific tissues themselves altering blood lipid composition and systemic metabolism. Moreover, there are likely to be regional variations in the metabolic response across tissues such as heart and liver, which cannot be studied using cell culture methods.

We will use some cell culture models, for example to test the effects of specific lipid species on metabolic function. This will not capture the complexity of inter-organ metabolic signalling in the body, but is adequate for the mechanistic analysis of lipid signalling on metabolic function.

We will use clinical samples from patients, and data from large cohort studies to probe associations between lipid species and metabolic function. This work is important but can only identify associations between different factors. To establish cause and effect, or to reveal the step-by-step mechanism of a metabolic process, we need to use animals and to intervene in these processes through the use of drugs or genetic modifications.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 address the scientific questions here, we estimate that we will need to run up to 20-25 separate studies. Each of these studies will typically include between 2-8 groups of animals (including appropriate control animals). Previous work in our laboratory has suggested that between 8-10 animals per group is a suitable number that is statistically-meaningful but not excessive. Some studies, however, will be smaller, pilot studies, which are not necessarily designed to optimise the statistical outputs, but to optimise e.g. drug doses, and these studies will typically use far fewer animals. Working on the basis of these numbers above, and equal numbers of rats vs. mice, we estimate that around 500 of each species will be used. These numbers are a little higher than those used on our previous licence, however, our research team has expanded and we have more questions we would like to address on this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As we design individual experiments, we carefully consider which control groups are necessary in order to address the scientific question, and we use power calculations to ensure that we are using an appropriate number of animals. We will make use of the NC3R's Experimental Design Assistant when designing such experiments.

Where relevant, factorial experimental designs will be used, rather than the one-thing-at-a-time approach. We consider carefully how to get the most out of each animal we use. For

instance, many of our animal studies involve the generation of a model of metabolic disease (often reflecting a human condition such as diabetes, obesity or fatty liver disease). The nature of these diseases is such that they affect many organs of the body. As such, if we plan an animal study designed to probe the effects of diabetes on the heart, for instance, we think carefully about which other researchers/students in the laboratory can carry out work on other organs such as liver and adipose tissue from the same animals in order to address questions of relevance to their work. This reduces the numbers of animals used overall, increases the amount of data obtained from a single animal and allows us to examine links between different tissues/organs, thereby enhancing the quality of the science produced.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will only set up our own breeding colonies if necessary, to avoid the wastage associated with a small colony.

We enthusiastically use pilot studies to minimise the number of groups necessary to address a scientific question. In particular, we find pilot studies to be particularly important when optimising the dose/route of administration of a new compound. For instance, a small, pilot study looking at different doses of a mitochondrial agent allowed us to use a single, optimal dose/route in a larger study, greatly decreasing the number of animals that would otherwise have been used. We estimate that use of a pilot in this case reduced our overall animal usage by 30-40 rats.

At post-mortem we routinely collect and store all tissues relevant to our overall programme of work to ensure that the minimum number of animals are used across our studies (for example, keeping samples of different skeletal muscles to complement cardiac muscle studies). Indeed this has been a key feature and a strength of our recent work, in that we have been able to carry out multiple high-throughput analyses on an individual animal's tissues post-mortem. As an example a recent paper from our group was the fifth peer-reviewed paper to be published using mouse tissue from a single study. By carrying out a single, well-designed study and banking the tissue, we were able to maximise the return from 140 mice, rather than using 700 animals in separate studies.

We also collaborate very effectively with other groups and industry collaborators. During the course of our previous project, we were able to reduce the number of animals we needed to use to address some of the questions by collaborating with a long-standing industry partner to analyse tissue that they had already planned to collect from a rat study of diabetes. This maximised the return of data from 108 rats used in their study, and reduced the number of rats that would have been used under our previous project licence by 40.

We will continue to use similar principles in our future work and look for new opportunities to reduce the number of animals we 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.

We will be using mouse and rat models of commonly-occurring metabolic diseases (e.g. obesity, diabetes, fatty liver disease). These will most often be produced by feeding the animals modified diets (e.g. high-fat or high-fat/high-sugar diets). Occasionally, we will need to study animals with more advanced forms of metabolic disease, and so this will involve working with animals with genetic predisposition to develop these conditions, or alternatively, we will treat some rats with a low dose of an antibiotic drug to alter pancreatic function and induce a mildly elevated blood glucose level. In order to assess the role of some key molecular regulators of metabolic function, we will also use some genetically-modified animals. In most cases, these models will cause nothing more than mild discomfort associated with obesity and perhaps more fatigue. This will be more pronounced in some of the diabetic animals, but will not be severe.

The methods we will use to study the nature of the metabolic alterations in these animals will rely on monitoring normal physiology - e.g. blood pressure, cardiac function, O_2 consumption/CO_2 production, activity. These techniques will cause not pain, but may rely on restraining animals, briefly anaesthetising them or single-housing them (usually for short periods, although in rare cases this may be up to 2 months). In all cases, we will carefully consider which of these methods we use on each animal to balance the desires to maximise the return of data and minimise distress to each animal.

We will need to inject some substances into the animals (e.g. drugs to modify metabolism or tracers to monitor metabolic flux). In all cases we will use the least harmful route of injection that is feasible for the substance in question. We will minimise the number of injections an animal will receive in its lifetime.

Why can’t you use animals that are less sentient?

Rats and mice are the animals of lowest neurophysiological sensitivity that are suitable for the study of mammalian metabolism. Since metabolism alters with development, and we are interested in metabolic diseases that progress over the course of a lifetime and occur in adults, we are not able to answer our scientific questions using immature animals alone. Some studies will begin in younger animals, which will be fed altered diets, but we will need to make measurements on them when they reach adult stages.

We carry out most of our analyses on organs and tissues post-mortem, however, in order to study the progression of metabolic diseases it is necessary to work with living animals and to monitor their physiology over time.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all studies we monitor animals frequently and where appropriate we use a scoring sheet, which is completed alongside frequent measurement (e.g. three times weekly) of body weight, food and water intake. Moreover, in our previous project we introduced a more detailed scoring system for monitoring clinical signs when administering agents to animals. Although the agents are administered at non-toxic doses, we find this scoring

system useful in understanding the interaction between such agents and e.g. hypoxic exposure, and sensitive to differences in the mild/moderate severity range. We felt this gave us a better framework to assess actual severity, whilst producing meaningful data which aided our interpretation of the biological response.

The outcomes of our project rely on the minimisation of suffering. Whilst we do not use any severe procedures, any change of environment could cause short-term distress. As such, all techniques that disturb the animal's environment or diet will involve acclimatisation periods such that data collected reflects true physiological changes due to a specific alteration, rather than generic stress. Blood pressure monitoring, for instance, will be undertaken following sufficient acclimatisation to the apparatus to minimise distress. Single-housing, for the purposes of measuring metabolic rates in a single animal, will be undertaken as infrequently as possible, and over the shortest period possible. Moreover, single-housed animals will be kept in proximity to their cage-mates and returned to group housing wherever possible.

Tissue samples will be collected from animals after they have been killed, with the exception of blood sampling from a superficial vein, which will be carried out on conscious restrained, or anaesthetised animals.

Animals will be handled during daily cage-cleaning and during studies where they will be weighed and their food/water intake assessed. This will habituate them to handling and restraint during procedures. Adequate nutrition will be provided during longer term dietary manipulation. Although there may be the occasional need to fast animals, the duration will be minimised. Animals will be housed with regular light/dark cycles (lights on for 12 hours, lights off for 12 hours) with temperature and humidity maintained in the facility and hypoxia chambers. Bedding will be regularly changed for all animals, and in particular will be carefully monitored in diabetic animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance on housing/husbandry, blood sampling and handling from the NC3Rs will be followed.

To anaesthetise animals for cardiac imaging, guidance from NC3Rs will be followed, with reference made to: Flecknell (2015) Laboratory Animal Anaesthesia, 4th edition, Elsevier.

Reference will be made to PREPARE guidelines (https://norecopa.no/prepare) when planning studies.

Reference will be made to LASA (https://www.lasa.co.uk/current_publications/) guidance, e.g. on record keeping, training, and avoiding mortality.

ARRIVE Guidelines v2.0 will be used when reporting studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PPL holder subscribes to monthly email updates from the NC3Rs and follows the NC3Rs on Twitter, and has encouraged all members of the research group to do so. We follow up by reading reports, and will join (and recommend) workshop webinars in future.

We regularly include papers of relevance to the 3Rs and our own research, in our group journal club, and openly discuss how we can implement the advances in our own projects. Moreover, we attend talks and sessions at meetings, that consider advances in the 3Rs.

The advances relevant to our research are often straightforward to implement, and by feeding back to the research group we are able to ensure that improvements made by one group member become more widely adopted. For instance, refined methods of handling mice have been successfully implemented in this way, with an experienced group member demonstrating improved handling techniques to others in the group, including new starters.

Home Office

167. Immune cell migration and function in inflammatory arthritis

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

inflammation, leukocyte trafficking, arthritis, netosis, therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project explores the process controlling the movement of immune cells from the blood into tissue during inflammatory arthritis; and it also tests the ability of new chemical therapies to restore normal immune cell movement and function during inflammatory arthritis and induce clinical remission.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 movement of immune cells from the blood into tissues is an important protective response to infection and injury. Once within the tissue the immune cells function to

destroy the infection and help to repair the damaged tissue. The movement and function of immune cells are very tightly controlled, like security checkpoints, to prevent unwanted inflammatory responses. However, many of these security checkpoints are lost in chronic diseases such as rheumatoid arthritis. Our work aims to provide more detailed knowledge on the factors responsible for maintaining these security checkpoints, and if certain new chemical therapies can restore the normal function of these checkpoints. Ultimately our aim is to use this knowledge to develop a new type of treatment that treats the cause of abnormal immune cell movement and function, rather than treating the symptoms that arise from unwanted inflammatory responses.

What outputs do you think you will see at the end of this project?

Project outputs will include:

advances in scientific knowledge on the processes controlling immune cell function and movement in health and in disease;

scientific and lay publications of our findings and methodology;

presentation of our data and/or methodology to the wider scientific community through conference oral and poster presentations;

identification of novel therapies that alter immune cell movement and function, which can be take forward to clinical trials.

Who or what will benefit from these outputs, and how?

This project has a wide range of beneficiaries.

Short-medium term

Scientists and innovations in analysing immune cell movement and function in health and inflammatory arthritis described in this licence will have an immediate impact on the ongoing projects at the host organisation, linked pharmaceutical collaborators and also further afield upon dissemination. Moreover, this project aims to improve our understanding the processes driving pathology in chronic disease, specifically linked to involvement of the chemokine-receptor engagement in regulation of systemic inflammation, an area of intense research interest worldwide. The impact of this is likely to occur in the short-to medium term, during the lifetime of this project and beyond as all publications are released.

Medium-long term

Collaborations – We intend to invite external seminar speakers who have an interest in immune cell movement and function, and inflammatory arthritides to our organisation, with the view of fostering further collaborations based on the concepts and ideas incorporated in this proposal. Additionally, we will continue to liaise with our pharmaceutical collaborators to expand on the work in this proposal, and further advance our knowledge and understanding of the drugability of target pathways and the therapeutic benefit of this for patients with rheumatoid arthritis. We envisage that these collaborations will occur during and following the completion of this project, and therefore represent a medium to long- term impact of this work.

Long term

Clinical Academics and the pharmaceutical companies involved in the project will benefit from advances in our understanding of disease pathology, along with our validation of new compounds in development. We are in continual conversations with these collaborators and view the translational of our findings into the clinic over the subsequent 10-15 years following the completion of this project representing the long-term impact of this project.

Patients - A key part of this licence is to test the efficacy of novel drug compounds prior to clinical trials for use of this drug in patients - as such a long-term aim of the licence is to provide new treatments for patients to improve their quality of life and potentially cure their disease.
How will you look to maximise the outputs of this work? Dissemination of information
We will work with the relevant teams at our Institute to facilitate communications and resulting impact. We plan to use several routes to disseminate our findings to the wider scientific community, industry and the public that will facilitate end-user engagement:

Peer-reviewed publication. We aim to publish high impact papers based on the findings generated from the research grants funding this project licence. In addition, our group has a strong tradition of publishing methodology papers; and negative data to ensure that groups do not unnecessarily repeat experiments that either technically are flawed or biologically yield the null hypothesis.

Presentations. We and our collaborators will present data at internal seminars along with national and international conferences, Dissemination via international societies. We and our collaborators are active members of various scientific societies, , allowing our findings to be disseminated to the wider scientific community in societal magazines and training workshops.

Enhancement of public understanding and engagement with research

We will take advantage of several events organised by the Public Engagement Working Group at our organisation and local charities to facilitate the public's awareness our research:

Science "pop-up" activities. We will give at least three research talks at student recruitment days and will be participate in "Meet the Scientist" tours. We will participate in the Annual Community Awareness Day and host an event local Science Festivals.

Lay Resources. We would develop lay resources, in collaboration with our patient/public research partners (PRP), for publication of Atlas of Science. Additionally, we will continue to involve patients and the public in the delivery and dissemination of research generated from this project.

Clinical Collaboration
My team and I are active members of several multi-institute research centers and will be able to present our findings at least twice annually at ongoing Centre seminars. We will also attend clinical conferences, where we will present our data and foster collaborative opportunities for translational research across the fields of rheumatology and chronic inflammatory diseases.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen the types of animals and their age based on our need to model a mammalian musculoskeletal disease that occurs in adult humans. As such, adult mice represent the lowest mammalian option available with a musculoskeletal system in which we can study immune cell movement in health and in disease, and to test potential drugs for human use. Importantly, we have selected the murine model of polyarthritis as this best reflects the clinical symptoms experienced by patients with Rheumatoid Arthritis.

Typically, what will be done to an animal used in your project?

Animals will have arthritis induced (100%), and test agents that might limit the movement or function of immune cells in tissues (e.g. the joints) will be given, and the tissues monitored during the inflammatory response.

The majority of animals (80%) will undergo general anaesthesia (up to 30 minutes), daily handling for scoring and caliper measurements of joints and limbs; administration of an agent (typically x14 days of oral administration), and will be killed up to week 10 via Schedule 1 method or non-schedule 1 for withdrawal of fluids (e.g. blood).

What are the expected impacts and/or adverse effects for the animals during your project?

All forms of arthritis cause joint stiffness, pain and some degree of disability. Animals may also lose weight or show signs of abnormal subdued behaviour. Arthritis, weight and behaviour will be monitored carefully using a scoring system that details when humane endpoints need to be actioned. Pain will be managed with pre-emptive pain relief.

We anticipate no adverse effects of the administered agents beyond those experienced during the administration step.

Expected severity categories and the 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 to approximately 95% of animals Mild severity to approximately 5% 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?

The steps leading to the development of the state of chronic inflammation are poorly understood and it is likely that multiple physiological processes are involved, including proliferation of precursor cells, activation of immune cells and recruitment of inflammatory cells to the site of inflammation.
Musculoskeletal diseases, such as rheumatoid arthritis, are not static diseases confined to a single tissue or time point and hence modelling disease onset and progression, along with the effects of new therapies inevitably involves the use of whole organisms and, in particular, the use of animal models of inflammation.

Which non-animal alternatives did you consider for use in this project?

We have pioneered a range of in vitro multi-cellular 3D constructs, incorporating primary human cells/tissues from healthy subjects and patients with different types of inflammatory arthritis. This has allowed us to further our understanding of the mechanisms regulating leukocyte recruitment and their effector functions. Indeed, for the majority of our work to date, we have used these in vitro tissue culture techniques to provide information about molecular and cellular mechanism(s) involved in the pathogenesis of disease or therapeutic effects.

Why were they not suitable?

Our in vitro models are unable to fully recapitulate the blood and lymphatic vasculature, and the movement of cells through complex organs, Moreover, new drugs must be validated in licensing authority approved animal models before they enter clinical trials and are tested on patients. In some cases, this work represents preclinical therapeutic (drug and/or cell) efficacy studies required prior to embarking on toxicology studies and human clinical trials. There are no other in vitro or in vivo alternatives to this work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used specific mathematical calculations based upon previous studies and the likelihood of our interventions producing positive results, to estimate the number of animals we will use in our study. For all experimentation, the lowest possible number of animals will be tested whilst ensuring that the experimental result is robust.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used statistical analysis to calculate the minimum number of animals necessary for this project.

We will continue to use the NC3R's experimental design tool to aid experimental design and consult trained statisticians before using any new protocols.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have used statistical analysis to calculate the minimum number of animals necessary for each experiment within this project.

Where new routes of administration or new interventions are being examined, pilot studies will first be established in 2-3 mice prior to full experiments. Subsequently these pilot data will used in the specific mathematical calculations described above to ensure that we use the minimum number of animals needed to obtain statistically significant results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in arthritis models affecting multiple joints that mimic the human condition of rheumatoid arthritis. This species and procedures have been chosen as they represent the lowest animal models with a musculoskeletal system in which it is possible to study immune cell movement in health and in disease, and the effects of human proteins have on this.

Why can’t you use animals that are less sentient?

Less sentient animals do not possess the same sort of skeletal structure that composes the joints, and often their vascular tree and immune system do not fully represent that of humans. Small rodents are the lowest mammals that can be used to recapitulate the human immune systems response to joint inflammation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Each experimental model will be monitored daily following intervention and mice will be assessed for any signs of distress such as pain and inability to feed. Barrier cream will be used prophylactically to prevent skin irritation from the agent used to induce arthritis. The mode of substance administration will be chosen to cause the least harm and distress to

the animal. Any new substances or route of administration will be tested in a small pilot study and the mice monitored daily for signs of distress.
Humane endpoints will be strictly adhered to at all times.

We have a very well refined, comprehensive and multifactorial scoring system specific for each individual polyarthritis model to capture the specific aspects of each arthritis phenotype, ensuring clear and consistent analgesia and humane endpoints. We have also made refinements to the housing of the animals to cater for any disability arising from arthritis - including soft flooring, non-tangling nesting material, long spouts on water bottles, food on the cage floor.

We will also systemically review each experiment on completion to see what lessons can be learned from the study in terms of endpoints (scientific and humane) and any animal welfare issues that may have arisen during the experiment that could then guide the subsequent experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Animal welfare is a key consideration in all of our protocols, and we will be guided by our NACWOs and NVS in always ensuring that we are using best practice and the most refined techniques. All staff involved in animal experiments will review the literature on animal welfare provided by the local AWERB. Following every experiment and regularly during group meetings we will review our procedures from a welfare standpoint to identify any potential for refinement.

We will also follow the published literature on arthritic models - for example the case for using different arthritis models to model the different aspects of inflammatory pathogenesis is extremely well made and described in Vincent et al. 2012. Moreover, refinement of the CIA injections has been described in Hawkins et al. (2015) and we have implemented these in our work to date and will continue to do so in this project.

If undertaking a systematic review, we will use SyRF, the free online platform for researchers, to perform a systematic review and meta-analysis of animal studies. This will allow us to keep up to date with any improvements in protocols and techniques which may reduce or replace the use of animals.

We will use ARRIVE2.0 when reporting animal work in publications, and PREPARE when designing experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to engage with Institutional efforts to promote the 3Rs and workshops; and receive the NC3Rs newsletter.

168. Targeting proteins involved in nucleotide synthesis in T-cell Acute Lymphoblastic Leukaemia

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Acute Lymphoblastic Leukaemia, nucleotide synthesis, drug target validation

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 determine whether proteins, identified in vitro as being involved in proliferation of T-cell Acute Lymphoblastic Leukaemia, are also crucial during the in vivo development of leukaemia, and therefore represent potential therapeutic targets.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

T-cell Acute Lymphoblastic leukaemia is an aggressive disease that occurs in adults and children. Approximately 50% of adults and 10-15% of children do not respond to the current therapy or relapse. There is no good alternative treatment for these patients and the vast majority of these will die. It is therefore important to identify new therapeutic targets. We have identified one such target (Excitatory Amino Acid Transporter 1; EAAT1) using an in vitro cell line approach and are now at the stage where we need to translate

our in vitro findings to an in vivo animal model.

What outputs do you think you will see at the end of this project?

At the end of the project we will be able to confirm whether the potential therapeutic target that we have identified in previous experiments is important in the pathogenesis of human T-cell Acute Lymphoblastic Leukaemia (T-ALL) in vivo. Following on from these results we will publish our findings and aim to develop drugs that will inhibit the function of the identified therapeutic target with the ultimate goal to contribute to curative treatment of T-ALL.

Who or what will benefit from these outputs, and how?

The findings from these experiments will show the in vivo role of EAAT1 in the development of T-ALL. Initially, through discussions this will benefit collaborators. The results will be disseminated through scientific publication in high impact journals and presentation at scientific meetings, which benefits collaborators, colleagues and other members of the field.

Confirmation of our in vitro data will highlight EAAT1 as a potential drug target, will support the development of therapeutic agents that can inhibit its function, which benefits those involved in such development, for example pharma.

Ultimately, we hope that this will result in the use of EAAT1 inhibitors as a means to treat T-ALL, which would show clear patient benefit.

How will you look to maximise the outputs of this work?

The publication of the results in peer reviewed journals, while maintaining open access, assures that novel knowledge is shared in an efficient manner. In addition we will present our results in scientific meetings. If any of our experiments are unsuccessful, we will make this available as well, for example though an open access platform like BioRxiv.

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.

In order to study tumour progression we will use immunocompromised mice. This is required because wild type mice would reject the cells, so tumour formation would not occur. Due to the requirement to inject the cells into the mice and the time it takes for T-ALL development to progress, we need to use juvenile to adult mice.

Typically, what will be done to an animal used in your project?
Animals will be injected with tumour cells to start engraftment of leukaemia cells. The leukaemia is then allowed to develop, at which point we will induce changes to the

expression of specific proteins. This will require the addition of doxyxycline to the food. We will then monitor through blood sampling and/or non-invasive in vivo imaging how the T-ALL development responds. At the end-point of the experiments the animals will be sacrificed using a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

During the early experimental stages the majority of mice will experience mild discomfort caused by the administration of injections, and/or monitoring of tumour burden. The time it takes an animal to fully develop leukaemia will vary, depending on the cells that are engrafted, with cell lines potentially only taking 3 weeks and primary patient samples estimated to take around 12 weeks. By monitoring leukaemia development, where possible we aim to end experiments before the animals exhibit adverse effects such as abnormal breathing patterns, an abnormal gait, or intermittent diarrhoea; therefore, body weight as well as body condition scoring will also be used to monitor clinical signs.

Expected severity categories and the 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 moderate, where animals with full tumour development are the ones that will experience the moderate classification. For this reason, 100% of animals may experience this severity, although we hope that our treatment groups will have reduced tumour burden. Furthermore, by monitoring leukaemia development we will aim to end experiments before reaching this 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 proposed work is based on in vitro work using cell lines. In order to confirm that our findings, relating to the function of proteins in leukaemia development, are also relevant in vivo, we require to perform this in human T-ALL cells within the whole organism. As we want to translate our work to human applications, we require a mammalian system where we can engraft human T-ALL cells.
Immunocompromised mouse models are most appropriate for this.

Which non-animal alternatives did you consider for use in this project?

We always perform experiments first using in vitro cell cultures with cell lines and/or primary cells. This is an alternative, but normally more a starting point. It was such in vitro work that led us to the identification of a therapeutic target. Now, for final validation that

this therapeutic target is indeed vital for T-ALL proliferation, human T-ALL xenografts in mice are the golden standard and as such there are no non-animal alternatives at this stage.

Why were they not suitable?

In vitro studies are an alternative, but cannot reflect all aspects of tumour biology, such as tumour microenvironment and physiological homeostasis. Therefore, we only take the modulation of gene expression forward to animal experimentation after they have shown efficacy in cell culture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have defined how many mice we need for each treatment group, including controls, and how many groups are needed to reach a conclusive result, avoiding experiments that need to be repeated due to inconclusive results. We will perform calculations using published data and data obtained by colleagues under similar experimental set ups to determine the number of animals required. Where appropriate we will include pilot studies to determine the variation between animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

By planning the experiments well (utilizing guidelines such as PREPARE), we will be able to ensure that we are not using more animals than required. This includes taking advice from research groups performing similar experiments and online tools, such as the NC3R Experimental Design Assistant. The use of methods to monitor the leukaemia development, such as blood sampling or in vivo imaging, will result in less variability as animals are their own longitudinal control and avoids the need to cull animals to measure disease progression.

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 and experimentally suitable, we will perform a pilot study on only 2-3 mice to ensure we can adjust our experimental design to use the minimum number of animals. At the end point we will collect as much data and tissues as reasonable, which can then also be shared with other research groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use immunocompromised mice. These are the model used for leukaemia engraftment as they do not reject the tumour cells. There are no established alternative models in which we can recreate the tumour microenvironment and physiological homeostasis.

Although there is no alternative approach, we ensure that the model is as refined as possible including the use of score sheets and monitoring of disease progression.

Why can’t you use animals that are less sentient?

Leukaemia engraftment and development can only be performed in the juvenile to adult stage of a mouse's life. Tumour progression takes time as well. Therefore, we cannot use immature mouse stages that are less sentient, nor can this happen under terminal anaesthesia. As we require a functional tumour microenvironment and physiological homeostasis for the human T-ALL cells to develop, we need a mammalian system and cannot use less sentient species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will closely monitor the animals undergoing procedures and especially towards the further development of the tumours. Tumour burden will be kept to a minimum within the requirements for the experiment. By monitoring disease burden through the use of blood sampling and/or in vivo imaging, wherever possible we will terminate the experiment before the animal develops signs of stress and discomfort.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the guidelines set out by the Laboratory Animal Science Association (LASA), as well as the PREPARE and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay aware of any advances related to the 3Rs through (1) information available from our licenced establishment, (2) keeping up to date with related research publications, (3) the NC3R website, which contains information as well as on line tools, (4) other information sources, like other research groups.

169. Investigating potential therapeutic agents using models of gastrointestinal cancer

Project duration

3 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

Cancer, Gastrointestinal, Therapy

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 test potential therapeutic compounds and drug candidates using in vivo models of cancers in the gastrointestinal tract (both established and pre-cancerous). Compounds may be given at different stages of the disease model to determine whether the compound modulates disease development, at what stage, and how.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 3rd most diagnosed malignancy and the 4th leading cause of cancer- related deaths in the world. Its burden is expected to increase by 60% to more

than 2.2 million new cases and 1.1 million cancer deaths by 2030. CRC is the 4th most common cancer in the UK and takes up 12% of total cancer cases in 2015-2017 (42,317 new cases), affecting men more than women.
Although CRC generally develops after the age of 50, young onset of rectal cancer is increasing. Studies also have found that young-onset CRC is often more aggressive and more likely to be diagnosed at an advanced stage than CRC in older populations.

Treatment options for gastrointestinal cancers depend on the stage and location (localised options include surgery and radiation, whilst systemic approaches involve chemotherapy, targeted therapies, and/or immunotherapy). If the cancer is confined to the location, surgical resection with removal of the cancerous mucosa/area is the most common treatment. Additionally, targeted therapies with a newer group of medicines that enhances the effectiveness of chemotherapy and prevents the cancer spreading can be integrated into the treatment.

CRC is treated using a combination of these treatments, however bowel resection to remove a tumour can require a stoma which impacts upon quality of life, and despite all efforts, the overall 5-year survival rate for CRC is only 65% (although early detection of CRC does improve the chances of survival). This low cancer survival rate mainly results from the limited options and availability of therapeutic drugs as well as serious side effects in the treatments.

Thus, there is an urgent need to identify new drug candidates that can either prevent polyps (i.e. early stage tumours) developing and/or reverse the development of pre-existing polyps.

What outputs do you think you will see at the end of this project?

As a result of the work under this project we expect to identify and characterise compounds that have therapeutic potential to reduce polyp formation and progression to tumours and/or treatment of gastrointestinal cancers. This information will be used to develop therapies.

Who or what will benefit from these outputs, and how?

Short-term: Initial studies will confirm compound safety and detection of activity to reduce polyp formation and progression to tumours

Medium-term: Further studies will confirm activity and determine compound pharmacokinetic properties and identify the effective ranges of dose and schedule.

Long-term: To generate a robust data package to be included in the preclinical section for regulatory paperwork to initiate clinical trials in UK or USA.

How will you look to maximise the outputs of this work?

The data generated directly from this PPL will have commercial value and so will not be immediately disseminated. However, any successful outcomes will be disseminated as part of the commercialisation process, and unsuccessful approaches will be published whenever possible.

Species and numbers of animals expected to be used

- Mice: 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.

Animals used for this work need to develop tumours in the gastrointestinal tract. Whilst older animals will naturally develop tumours, these are sporadic and occur throughout the body. A better approach is to use animals that have a genetic alteration (change in their DNA) which means they develop tumours in a predictable manner in the required region.

Mice are therefore used for this research as they are the species for which the most genetic alterations are available. They have also been widely studied for this purpose and so this prior knowledge can be used to inform project direction and animal numbers.

Juvenile mice may be used where compounds need to be administered prior to polyp (early tumour) development, but in all cases, mice need to progress to adult stages in order to allow the tumours to develop to a sufficient size for study.

Typically, what will be done to an animal used in your project?

The majority of mice are genetically altered to develop tumours in the gastrointestinal region (with a minority being wild type, i.e. 'genetically normal' when required as controls).

All mice will undergo oral gavage of a compound (or control) to determine whether the compound reduces/prevents tumour development (i.e. has therapeutic potential). Gavage will be given up to 5 days per week for a maximum of 10 weeks. The point at which the compound is first administered may vary in order to determine if the effects of the compound differ according to when it is administered relative to stage of tumour development.

Blood samples may be taken from some animals to measure the levels of compound and other markers in the blood.

Mice will be killed at the end of the study using a humane method of killing. They may be put under a terminal anaesthetic (i.e. will not be allowed to recover) for a final larger blood sample prior to humane killing.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically altered animals in which tumours are developing will experience the associated adverse effects. This includes developing anaemia during the early stages (from around 12 weeks). Whilst not causing adverse effects in itself, this results in pale feet, which is used as an indicator for disease progression. Mice then gradually lose weight (from around 4 months).

All animals will undergo oral gavage, which causes transient discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

100% of mice are expected to reach a moderate severity. However, the hypothesis is that the compounds administered will reduce/prevent tumour development. If correct, then the severity may be lower in some ofthese 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 aim of this project is to establish whether the compounds administered have a therapeutic effect on the development of gastrointestinal tumours (i.e. that they reduce or prevent tumour development).
Whilst highly predictive human patient-derived and mouse 3D in vitro “mini-gut” organoid models are extensively used to identify and characterise compounds, ultimately the current non-animal methods do not replicate the complexity of tumour development within a whole body system which involves dynamics such as a circulatory system. Animals are therefore required to ensure that the compound elicits the predicted effects when given via clinically relevant routes.

Which non-animal alternatives did you consider for use in this project?

"Mini-gut" (organoid) models are a 3D in vitro approach that recapitulates the intestinal mucosal cell production of healthy and diseased tissues in humans. They are therefore one of the closest technologies to using a whole body system and can be created using cells from either patients or mice.

Compounds under investigation in this PPL were discovered and characterised using organoids generated from cells taken from patients suffering from inherited familial adenomatous polyposis; FAP. These patients carry an inherited mutation in the gene APC that initiates the development of colorectal cancer. Having characterised compounds in the patient-derived organoid model, the activity was then confirmed using organoids generated from mouse mucosa cells that carry the same mutation ($Apc^{Min/+}$).

Why were they not suitable?

Despite the highly predictive nature of the “mini-gut” organoid models, and their successful use in demonstrating that the compounds under investigation are highly likely to elicit a therapeutic response, ultimately the compounds need to be validated in a whole animal

model prior to entering clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers have been estimated based on pilot data, prior experience of these animal models, and published data. This information has been used to undertake calculations in order to estimate the number of animals required in each experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments have been designed according to best practice in experimental design, for example ensuring animals are randomly allocated as experimental or controls, and that animal care staff and researchers are unaware of which group the animals belong to when performing procedures and analysis wherever possible. This minimises any bias that may be introduced which would result in more animals being required, or incorrect conclusions being drawn from the results.

The NC3Rs' Experimental Design Assistant will be used to support the design of complex experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where in-house or published data does not exist, pilot studies will be undertaken to provide the information required to more accurately calculate animal numbers for experiments. The data obtained from each animal is being maximised by ensuring that tissues are harvested post-mortem wherever possible, so if a compound is shown to have an effect on tumour development, additional tissues are available to investigate how the compound is having this affect, minimising the need to repeat an experiment to obtain other samples.

Control groups will be shared across experiments as much as possible, assuming that this does not impact on best practice in 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.

This project uses genetically altered mouse models that develop tumours in the gastrointestinal system. They will be administered with compounds with demonstrated activity in highly predictive human patient-derived and mouse 3D in vitro “mini-gut” organoid models as candidates for therapies to reduce or prevent tumour formation or growth.

The genetically altered mouse models are selected so as to cause the least pain, suffering, distress, and lasting harm, whilst being appropriate for answering the scientific question.

These models are widely used and published in the literature. This information has been used to develop bespoke welfare score sheets that aid in the monitoring of pain, suffering, and distress. This ensures that humane endpoints are as accurate as possible and can be detected and acted upon quickly.

The volumes and frequencies of administration of compounds (and controls) are as refined as possible whilst still scientifically and clinically relevant.

In addition, animals will receive supportive husbandry measures such as the use of refined handling techniques and the provision of soft mash as appropriate.

Why can’t you use animals that are less sentient?

Small animal models are the least sentient species required to validate the compound prior to Phase I clinical trials, and so a less sentient species unfortunately cannot be used. Due to the timeline of tumour development, we cannot use mice at a more immature life stage, and nor can we work with terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The models used in this project are established and widely published. We use bespoke scoring sheets that include any early markers of disease progression (such as pale feet) to help monitor animal welfare.

The volumes and frequencies of compound administration are selected to be the most refined whilst scientifically justified. For example, flexible gavage needles are used to minimise potential tissue damage, and needles may be dipped in sucrose to aid in reducing stress associated with this route.

Wherever possible, mice will be handled using the refined handling techniques to minimise stress more generally.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All volumes administered will be according to LASAguidelines.

Blood sample volumes and frequencies will be according to LASA guidelines.

The NC3Rs' ARRIVE 2.0 website will be consulted at the start of experiments to ensure that all elements are considered. Any resulting publications will be in accordance with the ARRIVE 2.0 guidelines.

The PREPARE guidelines may also be consulted at the start of any experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of my organisation's 3Rs group and AWERB. I also regularly receive updates on the latest advances in the 3Rs from both the NC3Rs (via their newsletter and other updates), and also other sources (such as the various online forums and other members of the focus group).

Home Office

170. Examining SLFN14 function in haemostasis and thrombosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

platelets, haematopoiesis, red blood cells, haemostasis, thrombosis

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine how the novel gene/protein SLFN14 regulates haematopoietic cell development and function of platelets and red blood cells/erythrocytes and how this gene/protein leads to excessive bleeding 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?

Reduced numbers of platelets and abnormal red blood cells are associated with excessive bleeding in humans. By understanding how platelets and red blood cells are produced we can identify new targets for drug development. A drug that can prevent excessive bleeding that occurs in these patients will save lives.

What outputs do you think you will see at the end of this project?

Reduced numbers of defective platelets and abnormal red blood cells are associated with excessive bleeding in humans. By investigating the novel gene/protein SLFN14, this will lead to us to understand how platelets and red cells in the blood are produced.

Who or what will benefit from these outputs, and how?

Human patients whom experience excessive bleeding will benefit from this research. Overall this research will help to inform patient management and lead to more specific and tailored treatments for the future. The information gained from this project will likely benefit patients within 5 - 10 years as below:-

Short term: colleagues and collaborators benefit from data directly generated Medium: further work to validate as potential therapeutic target
Long term: drug development/pharma benefits if shown SLFN14 or member of pathway are potential therapeutic targets

How will you look to maximise the outputs of this work?

The current associated funded Programme grant is already a result of a successful collaboration with a research group in New York, USA. Also other 'spin-off' funding applications will also be considered in the near future. Any specific patient-based information is fed back to the patient. Publications gained as a result of this related research (but also unsuccessful/negative findings) are always communicated within the research community but also to an audience more widely such as conferences to scientific and clinical communities.

Publication of the data in appropriate journals will be done using the ARRIVE guidelines on reporting in vivo experiments.

Species and numbers of animals expected to be used

- Mice: Over 5 years, we would expect to use no more than 8500 mice in total - 2500 animals for the experiments and 6000 to breed the genetically altered strains required

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Where possible human platelets and red blood cells will be studied, however in order to decipher more specifically the role of SLFN14 mutations, we need regular samples which possess these mutations.
We cannot obtain tissue from patients as regularly as we require therefore mouse models are necessary to provide us with sufficient material to address our hypotheses in the longer term. We cannot use non-mammalian species for this work as mammals are the only animals to have platelets.

In vitro techniques involving mammalian platelets and red blood cells are hampered as they do not have nuclei and so cannot be genetically modified. Furthermore megakaryocytes and erythroid progenitors (platelet and red blood cell precursors) are extremely difficult to isolate in large numbers from human patients. These involve invasive procedures which are not appropriate on a regular basis in patients with poor platelet function and whom already require extensive treatment for their condition. Adult mice are required due to their ability to produce platelets and red blood cells from progenitors in the bone marrow as opposed to fetal liver and spleen at younger age points. Haematopoiesis in the bone marrow is more relevant to the disease manifestation and progression in our human patients thus supports the need for adult mice in our study.

Typically, what will be done to an animal used in your project?

Mice will be bred to carry specific genetic mutations in the SLFN14 gene. Typically, mice will be subjected to injections to alter platelet production and blood samples may be taken from some mice according to guidelines. Larger blood samples and bleeding assays will be performed under terminal anaesthesia when required.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect our genetically modified SLFN14 mouse models to mimic the mild blood cell (both platelets and red blood cells) phenotypes observed in human patients with SLFN14 mutations. The heterozygous SLFN14 mutated mice have a phenotype of anaemia and platelet dysfunction but this is only mild and will only manifest during bleeding/clotting assays, which will be performed under terminal anaesthesia. The animals will be checked regularly for signs of bleeding, pain and distress, and will be culled if these symptoms or signs of excessive bleeding occur. Transient pain may also result from blood sampling and administration of 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)?

Protocol 1 (breeding): 90% of genetically altered mice will have mild severity or less.

Protocol 2 (bleeding and clotting assays): a third of animals will experience moderate severity, with the remaining two thirds experiencing mild severity.

Protocol 3 (non recovery bleeds): all animals will experience 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?

The fundamental reason why the use of protected animals is required to understand these processes is that at present no in vitro methods exist to model platelet or red blood cell function from primary cultured cells. Platelets and red blood cells lack a nucleus and as such, cannot be cultured or manipulated to possess mutations in SLFN14 that we require in our model. Megakaryocyte primary cell cultures, or immortalised megakaryocyte-like cell lines do not produce sufficient numbers of platelets of the same level of reactivity as in vivo.

Which non-animal alternatives did you consider for use in this project?

Where possible, in vitro experiments in cell line models alongside studies in patient derived samples, have been used to investigate the mechanism of action of SLFN14 and its regulation, and its role in platelet aggregation and formation.

Why were they not suitable?

These studies are limited due being unable to obtain large volumes of patient material. Cell line studies have a different complement of proteins and give no functional information, which can only be done in ex or in vivo progenitor cells, platelets and red blood cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The major use of animals is in the generation of genetically altered mice and their litter-matched controls. The experimental design involves undertaking a series of studies to investigate cellular and functional processes of genetically altered platelets, red blood cells and bone marrow progenitors in response to stimuli. The majority of these studies will give clear-cut answers from between 3 – 5 animals, although in some situations, we may need to increase this value. For example, when more than one gene contributes to a pathway (the effect on loss of one gene may be relatively small) or where tests are known to be associated with wide variation such as flow-based studies on collagen surfaces. For studies to investigate haemostasis and thrombosis in mice, we will use established statistical tests to determine the statistical significance of a result. Where appropriate, we will use power calculations and will consult with an external statistician to ensure that we are using the appropriate number of mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Reduction will be achieved by first performing in vitro experiments on primary megakaryocytes, iPSC cell lines and human patient platelets, using gene manipulation techniques such CRISPR and shRNA to identify the mechanism through which SLFN14

regulates megakaryopoiesis and platelet function.

We maximise the number of tissues we can obtain from a single animal and can store platelets, red blood cells and progenitor cells from the bone marrow from a single mouse for later biochemical studies. Thus, we are able to keep the numbers of mice to the minimum required to address a particular question whilst conducting multiple assays. For example we answer more than one research question with intravital microscopy where multiple cell types are involved. We have a lot of previous experience using these assays and experiments in our research group.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our experiments require the intercrossing of a number of strains in order to generate mice carrying the required genetic alterations. This potentially requires large numbers of animals, however we will use breeding strategies which generate the required genotypes using the fewest mice possible.

We will use established parametric and non-parametric statistical tests to establish the statistical significance of a result depending on the nature of the data. Where appropriate, we will use power calculations to ensure that we are using the appropriate number of mice. We will also optimise the number of animals by maximising use such as taking multiple samples from a single mouse e.g. Blood, bones and spleens.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models used

We will be using genetically altered strains which are needed because they mimic the human heterozygous condition (platelet and red cell dysfunction) studied in this project. Where possible we will use breeding strategies to avoid generating null/homozygous mice unless needed scientifically.

Choice of methods

Blood collection is carried out either under terminal anaesthetic, or in the case where small samples are required, according to LASA guidelines.

Substances will be injected to alter platelet function. They will be injected via the most refined whilst scientifically justified route, according to LASA guidelines.

Bleeding and clotting assays will be performed under terminal anaesthesia, with all tissues

collected postmortem.

Why can’t you use animals that are less sentient?

We cannot use a non-mammalian species for this work, as mammals are the only animals to have platelets. In mice, there is established and reliable transgene technology, and established models of platelet function. There are a large number of genetically modified mutants that are available and there is an extensive amount of work that has already been performed and published using mouse models of thrombosis and haemostasis. In addition, mice are shown to have a high similarity of their SLFN14 protein to humans making the use of mice particularly relevant and translatable to our human patients. Where possible we will use mouse embryos, such as for fetal livers which are required for megakaryocyte culture.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Further refinement of studies during the course of this project

During the protocols, we will monitor the health status of the animal during all procedures in this licence, including in vivo models and bleeding requiring terminal anaesthesia.

For all procedures, we will continually monitor the literature for methods of refinement and we will review the literature for new developments to ensure that the use of animals continues to be necessary.

Where appropriate each animal will receive analgesia which is not anti-inflammatory in nature to reduce pain and distress. As this is a new GA strain animals will be monitored for emerging bleeding phenotypes.

We will use refined animal handling techniques, use reagents with published protocols where possible and pilot studies to assess a method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to ARRIVE and PREPARE guidelines. Additionally the latest best practice guidelines published by Laboratory Animal Science Association (LASA) (www.lasa.co.uk/publications.html.
Additionally for the latest information I will check the requirements by liaising and working closely with our local Animal Welfare and Ethical Review Body (AWERB) who share good practices. These should all help to improve animal welfare and the quality of science; further the application of all 3Rs, and promote a ‘culture of care’ at all establishments where animals are bred, supplied or used for research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

For all procedures, we will continually monitor the literature for methods of refinement and consider whether the use of animals is necessary to address the experimental question under investigation.

We will also attend conferences and meetings from the NC3Rs organisation and BMSU, as well as keeping up to date with all the latest email alerts and regulation changes that are communicated.

171. Enhanced Bone Repair

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

Bone, Repair, Regeneration, Enhancement, Defects

Animal types	Life stages
Rabbits	adult
Sheep	adult
Pigs	adult
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?

Bone defects are serious conditions in which a part of the bone is damaged or missing owing to either trauma or surgery, and need to be repaired through interventional techniques such as bone grafting or bone void fillers. Consequently, the primary aim of this project is to develop novel therapeutics and surgical techniques to facilitate fracture repair and replace bone that is lost due to either disease, trauma or surgery with the support of *in-vivo* models such as the calvarial defect, long bone or segmental defect, partial cortical defect and cancellous bone defect models to help screen the efficacy of novel devices/products.

These experimental models for studying bone repair need to reflect the biomechanics and the physiology of the particular clinical scenario in humans. The therapeutic materials may be either (a) synthetic, i.e. polymeric (resorbable or non-resorbable scaffolds, or a combination of both), metallic or ceramic or biologic (growth factors, cells, signalling

molecules, placental tissue and bone marrow aspirate).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Bone regeneration, i.e. improved fracture and bone defect repair is a key strategic area of research. Under previous licences, we have used bone repair *in-vivo* models to help provide safety and efficacy data that has been used to support the development and launch of new products. Furthermore, the project licence provides an opportunity to expand indications of products acquired through strategic acquisitions. This approach further emphasizes our desire to expand into regenerative medicine and extremities with access to new biological therapies. Our current R&D activities also include the assessment of polymeric materials, cells and pharmaceuticals to enhance bone regeneration. Degradable polymers, for example, negate the requirement for a second surgical intervention to remove metallic hardware. Such polymers can be used as ‘internal splints’ for long bone fracture fixation or as ‘bone adhesives’ to aid in the repair of bone fragments.

What outputs do you think you will see at the end of this project?

The primary output of this project is to develop new products/methods to treat bone defects and/or enhance bone repair using existing experimental *in-vivo* models developed under previous licences. Additionally, this will help gather scientific knowledge and generate publications to better the wider scientific community.

Although, the current set of *in-vivo* models have shown to translate clinically, there is still an opportunity to expand and increase the clinical relevance of the models available. Therefore, a secondary objective will be to develop new experimental *in-vivo* models for studying bone repair that better reflect the biomechanics and the physiology of the particular clinical scenario in humans, which results in bone loss e.g. delayed union; established hypertrophic nonunion (abundant callus formation); established atrophic nonunion (little to no callus formation); fractures with a segmental defect; fractures at risk of delayed or nonunion (permanent failure of healing), i.e. high-energy and open fractures, infected fractures and fractures in compromised patients. These new models will in turn, facilitate the development of innovative products, which will improve patient outcomes.

Who or what will benefit from these outputs, and how?

SHORT-TERM BENEFITS (0-3yrs): Although, the current set of *in-vivo* models have shown to translate clinically, there is still an opportunity to expand and increase the clinical relevance of the models available. These new models will in turn, facilitate the development of innovative products, which will improve patient outcomes. The project will attempt to develop new experimental models of bone repair, which will help advance the frontiers of science in this area for the benefit of clinicians appraising preclinical trauma studies and researchers investigating compromised bone healing or novel treatments for fractures. It will also allow us to investigate new indications for products obtained through mergers and acquisitions, which could be adapted to enhance bone repair. These therapeutics will be tested using existing bone repair models developed under previous

licences.

LONG-TERM BENEFITS (5-10 years): The project will also provide long term benefits for patients undergoing limb reconstruction as a result of trauma or surgery using new therapeutics and surgical techniques to salvage severely injured limbs with bone defects in the acute phase of treatment that were not previously attempted. Attempting limb reconstruction in the presence of significant bone loss usually involves surgery, which is technically difficult, time-consuming, physically and psychologically demanding for the patient, and with no guarantee of a satisfactory outcome. The function of the salvaged limb may be disappointing due to residual pain, joint stiffness and neurovascular deficit. The patient may require a secondary amputation due to refractory infection or non-union. The ability to develop additional therapies to address bone loss will improve the overall management of these patients.

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

- Rats: 300
- Mice: 100
- Rabbits: 140
- Sheep: 380

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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: Sheep have been selected as a large animal experimental model given that their anatomical size and skeletal dimensions is similar to humans, and for ease of supply.

Skeletally mature sheep are preferred over juvenile and aged animals in this licence for the following reasons; (a) availability from the open market, (b) our current implants and surgical instrumentation also tailored towards the bone of skeletally mature animals, (c) bone repair and remodeling processes are dissimilar between juvenile and skeletally mature animals and so juvenile animals are not considered to be sufficiently representative of the clinical conditions under study. From past experiences, aged sheep are more susceptible to succumbing to complications arising from the surgical procedure and the effects of the general anesthetic. Therefore, for this reason young adults are more suitable.

RABBITS: Rabbits have also been selected as a small animal experimental model. The rabbit model is a useful intermediate prior to progressing to the larger animal model, which may be a better representation of the clinical setting. Skeletally mature rabbits are the

preferred choice for this licence due to (a) the ease of supply from registered breeders and (b) their bone remodelling process is more representative to adult humans than juveniles where growth plate fusion has not occurred.

RATS: Rats have also been selected as a small animal experimental model given their long history of use in the literature. Skeletally mature rats are the preferred choice for this licence due to (a) the ease of supply from registered breeders and (b) their bone remodelling process is more representative to adult humans than juveniles where growth plate fusion has not occurred.

MICE: Mice have also been selected as a small animal experimental model given its long history of use in the literature. Mice are the preferred choice for this licence due to (a) the ease of supply from registered breeders and (b) their bone remodelling process is more representative to adult humans than juveniles where growth plate fusion has not occurred.

Typically, what will be done to an animal used in your project?

Surgically, under general anesthesia with recovery, a defect will be created in the bone. Test or control materials are then inserted into the defect or the defect is left empty as a control treatment. The estimated time of the surgical procedure is approximately 1 to 1.5hrs. Once the animal has recovered from the effects of general anesthesia it will be monitored by qualified animal husbandry staff until the live phase of the study is completed. Live phases are typically up to 26 weeks but can be up to 3 years depending on the model used and the materials being evaluated. At the end of the study, the animals will be humanely euthanized.

What are the expected impacts and/or adverse effects for the animals during your project?

Typically, surgery will involve the creation of a bone defect, which may lead to some degree of discomfort immediatey after surgery. This will be reduced by the use of a minimally invasive technique and a controlled method for the creation of the bone defect. Any additional discomfort will be minimised with the use of 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)?

- **Rat:** It is expected that greater than 95% of animals will experience moderate harm. The remaining 5% or less of animals may be classified as non- recovery if they react adversely to an anaesthetic or are euthanised due to irreparable surgical complications.
- **Mice:** It is expected that greater than 95% of animals will experience moderate harm. The remaining 5% or less of animals may be classified as non- recovery if they react adversely to an anaesthetic or are euthanised due to irreparable surgical complications.
- **Rabbit:** It is expected that greater than 95% of animals will experience moderate harm.

The remaining 5% or less of animals may be classified as non- recovery if they react adversely to an anaesthetic or are euthanised due to irreparable surgical complications.

- **Sheep:** It is expected that greater than 95% of animals will experience moderate harm. The remaining 5% or less of animals may be classified as non- recovery if they react adversely to an anaesthetic or are euthanised due to irreparable surgical complications.
- Due to the enforcement of humane endpoints in each protocol, it is highly unlikely that any animals will suffer any severe 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?

Animals will only be used where there are no viable alternatives to the use of animals in order to answer the questions that the project requires.

Bone regeneration is a complex, well-orchestrated physiological process of bone formation involving signalling cascades and cellular repair mechanisms that cannot be studied by *in-vitro* cell culture studies alone where these complex interaction are impossible to truly replicate. Consequently, bone regeneration can only be demonstrated with the help of animal models, i.e. no *in-vitro* methods such as computer simulations or cell cultures can mimic the complexity of an *in-vivo* environment sufficiently or predict clinical efficacy. Bone defects are typically caused by various conditions, which challenge orthopaedic surgeons and biomedical scientists. The *in-vivo* models developed under this licence tailored for studying bone repair will attempt to reflect the biomechanics and the physiology of the particular clinical scenario in humans, e.g. fractures with a segmental defect, fractures at risk of delayed or non-union, i.e. high-energy and open fractures, infected fractures and fractures in compromised patients, e.g. immunocompromised, or osteoporotic whereby their natural bone repair processes are inhibited by their disease state.

In-vivo models will also be required when *in-vitro* systems cannot provide a reproducible approximation of the real-life *in-vivo* or clinical setting, e.g. the kinetics of delivery and distribution of drugs or bioactive factors, and the biocompatibility and degradation properties of implant materials.

Initial screening and feasibility testing will be carried out in rodent models whereas large animal models, whose bone regeneration is closer to the same processes in humans, will be used to provide translational proof of concept.

Regulatory authorities, e.g. Food and Drug Administration (FDA) and European Medicines Agency (EMA) will also require the testing of novel bone repair therapies in both a small and large animal model before accepting an agent for clinical trials to ensure

clinical translation in bone tissue engineering and regenerative medicine through assessment of appropriate efficacy and safety endpoints.

Which non-animal alternatives did you consider for use in this project?

In-silico bone mechanobiology will be used where appropriate for predicting mechanobiological changes to bone tissue and investigating cell mechanobiology. *In-vitro* cell culture models will be used for studying bone mechanobiology in 2D by applying controlled mechanical stimuli to relevant cell lines and also to establish safety of any technology prior to any *in-vivo* experimentation. Collectively, these tests will be used for screening out some of the early stage technologies.

Why were they not suitable?

These preliminary tests will be used to screen out novel technologies that lack supportive data. However, in order to provide a sound basis for making determinations about reasonable safety and efficacy, animal models will be required to provide accurate information about how a medical intervention will perform in a human clinical trial. Furthermore, regulatory authorities such as the FDA and MHRA will require evidence of *in-vivo* safety and efficacy data prior to clinical evaluation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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.
ISO standards (ISO 10993)

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. Typically, welfare studies to assess new procedures or technologies under this licence will consist of no more than four animals. 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.

To further minimise numbers, where possible, one-sided statistical tests in which the critical area of a distribution is one-sided so that it is either greater than or less than a certain value, but not both, 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 Animal Welfare and Ethical Review Body (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?

COMPUTER SIMULATIONS: Computer generated models of the anatomy will be generated from Computed tomography (CT) scans of isolated limbs to create 3D models that can mimic functions of physiology and help determine safe corridors for surgical implantation. These models will also be used to develop customized implants reducing the risk of any surgical complications.

***In-Vitro* Testing:** *In-vitro* cell culture models will be used for studying bone mechanobiology in 2D by applying controlled mechanical stimuli to relevant cell lines, and also to establish safety of any technology prior to any *in-vivo* experimentation

QUALITY ANIMALS/VETERINARY CARE/PRE-SCREENING: Radiological templating of the animal prior to surgery will help screen out animals that are deemed to be unsuitable for surgery due to either health reasons or unsuitable anatomy. The images collected can also be segmented to create 3D models to assist with implant development. The loss of animals can also be minimized by providing good post-operative care, avoiding unintended breeding, and planning ahead so that the appropriate number of animals needed for the studies are ordered and/or bred.

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 either new procedures or technologies 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, histology and biomechanical testing.

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.

A thorough investigation into the most relevant species for simulating bone repair have been obtained from the knowledge gained from previous work in our facility. The selected models and methods have shown that they cause the least amount of pain, suffering and distress to the animals.

INITIAL SCREENING: Rats, mice or rabbits will be suitable for the evaluation of technologies at an earlier stage of development prior to production of implants designed for humans. Our facility have accrued a great deal of experience with these animal species from previous programmes of work This experience has led to refinements in surgical technique, analgesic regimes and post-surgical care. Gait analysis has been used successfully to monitor recovery after surgery in sheep models and this analysis has been used to improve post-surgical care.

HUMAN IMPLANT SCREENING: Other protocols listed in this licence will utilise sheep as this species has bones of the size that are compatible with human implants and surgical techniques.

Why can't you use animals that are less sentient?

Live mammalian vertebrates are required that closely mimic the bone repair pathways and human skeletal system as much as possible to ensure that any data generated can be translated to the clinical situation.

The protocols assigned to the less sentient species (rat, mice and rabbit) are generally intended to be used for the initial/earlier stage assessment of technologies tailored for enhanced bone repair.
However, as the technology progresses through the product design control matrix, there will be a requirement to assess their safety and efficacy in animal species whose anatomy and physiology more closely represent the intended use in humans. These animals (sheep and pigs) tend to be of higher sentience.

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 macro- environment, (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, drug pumps).

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.

172. Supply of tissues

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Blood, Tissue

	Life stages
Mice	adult
Rats	adult
Rabbits	adult
Cattle	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?

To provide blood and other animal tissues to the research community for subsequent in vivo and ex vivo studies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The ability to supply extremely fresh living animal tissue (including blood) to research colleagues for subsequent ex-vivo studies is essential to minimise the impact of transport on tissue viability. Moreover, such use maximises use of any animals not required for specific research projects.

What outputs do you think you will see at the end of this project?

Successful production of biological products (to a high ethical standard) that effectively

and efficiently meet the demand of research colleagues. The resulting products being incorporated into in vitro and ex- vivo research programmes that will expand academic knowledge via successful publications in high profile peer reviewed journals or having a translational impact via development of new diagnostic and therapeutic approaches for disease conditions affecting both humans and other animals.

Who or what will benefit from these outputs, and how?

The academic community via publications arising from the studies undertaken using the products of this licence. This will likely be in the medium to long term.

How will you look to maximise the outputs of this work?

Research colleagues and their respective group members will publish work arising from the use of products obtained from animals used under this licence in scientific journals relevant to their respective areas of science.

Blood products supplied to local commercial research organisations will be used for in-vitro testing and active development of new medicines with potential therapeutic application.

Where tissue or blood products are being collected from naive animals, the potential for any secondary surplus material will be notified in advance (where appropriate) by the use of the 'in house' distribution/notification list and internal intranet notice board.

As each application for use of animals under this licence will be considered by members of the AWERB, each use of animals will be subject to more scrutiny and have greater oversight than may be the case at third party establishments. In turn, scientific members of the AWERB will be able to alert colleagues to any products of interest arising from protocols undertaken.

Species and numbers of animals expected to be used

- Cattle: 10
- Sheep: 10
- Mice: 3750
- Rats: 360

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 both species required by research colleagues to supply tissues and blood products for in-vitro or ex-vivo tissue experiments. The blood and tissues from these species are used extensively to develop new medicines and study biological responses ahead of any subsequent in-vivo animal studies relevant to humans and other animals.

Adult animals will be used as their larger circulating blood volume means that fewer animals are required.

Typically, what will be done to an animal used in your project?

The typical experience of animals undergoing a non-recovery protocol for the collection of blood would be exposure to an anaesthetic gas or the experience of an injection of an anaesthetic drug. The anaesthetic agents would be administered in such a manner as to minimise the risk of panic and the animals would gradually lose consciousness and enter a stage of deep surgical anaesthesia. At this point blood and/or tissues would be collected. The loss of blood during the procedure would ensure that the animals would be unable to regain consciousness; however humane killing would be completed and confirmed according to Schedule 1 of the Act.

Cattle or sheep will be prepared for blood sampling by being acclimatised to restraint and human contact and receive a food reward after each procedure. The area around the vein may be clipped and shaved and will be cleaned ahead of administration of local anaesthetic, if appropriate and where this does not add to the potential impact of restraint on the animal. The animals will experience the sensation of a needle insertion to allow collection of blood from the appropriate blood vessel located close to the surface of the skin. On rare occasions (<10%) a second attempt to obtain blood from another vessel may be undertaken if the blood vessels constrict rapidly following venepuncture.

What are the expected impacts and/or adverse effects for the animals during your project?

For animals undergoing blood sampling and/or tissue collection under terminal anaesthetic, the deep surgical anaesthetic plane will prevent animals feeling any pain.

When animals are blood sampled in a conscious state the refined techniques, in line with LASA and NC3Rs guidelines, will ensure that any pain is mild and transient in nature. On rare occasions <5% a bruise or swelling at the site of blood sampling may form which should subside within typically 3 to 5 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity of this work is Non recovery for 100% of animals blood sampled under terminal anaesthetic.

The expected severity experienced by animals undergoing blood sampling while conscious will be Mild. This will apply to 100% of animals on such protocols.
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?

Animals are required to produce the whole blood and tissues used for research undertaken either in vitro or ex-vivo. This means that experiments can be conducted without the requirement to conduct more invasive procedures on living animals, thereby replacing or reducing the overall number or severity of animal use.

Which non-animal alternatives did you consider for use in this project?

For each individual request to use this licence, AWERB will scrutinise the proposal to ensure appropriate consideration has been given to alternatives as well as reduction and refinement.

Why were they not suitable?

Suitability will be reviewed by AWERB on a case-by-case basis as each request is received.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 to be used during the five year duration of this licence are estimated according to likely demand for blood and other tissues.

The main use is to supply blood for in vitro research techniques used in the development of new medicines. Therefore by using current demand for rodent blood per month from existing licence authority (50ml mouse blood and 30ml of rat blood) and calculating the amount of blood that can be obtained per animal (by species) it is possible to estimate the likely number of animals required per month to meet this demand and then extrapolating for the five year duration of the licence.
For blood obtained by serial sampling from donor animals (sheep and cattle) it is estimated that 2 animals per year over five years will meet demand based on previous interest shown by research colleagues for this source of fresh blood.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Steps taken to reduce the numbers of animals used in this licence include building in the ability to re- use animals that have received minimal mild and transient interventions e.g. removal of blood via venepuncture.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where naive animals are used for blood collection under terminal anaesthetic, the possibility of sharing other tissues with research colleagues (after blood has been collected and death confirmed) will be explored. The facility electronic distribution list and internal intranet notice board can be used to alert colleagues in advance of the availability of fresh tissue for ex-vivo 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.

Adult mice, rats, sheep and cattle will be used to supply blood and other tissues for subsequent in-vitro or ex-vivo studies.

For the supply of large volumes of rodent blood the method that will be used is recognised as causing the least pain, suffering, distress or harm to the animal is where the animal is terminally anaesthetised to a level of deep surgical anaesthesia by a competent and trained personal licence holder. Once the animal is deeply unconscious, blood is removed directly from the heart by use of a needle and syringe. In addition, where fresh tissue is required, this is surgically removed while the animal remains deeply unconscious. The animal is maintained under anaesthesia for the duration of the protocol and never regains consciousness. Once the blood and/or tissue is obtained, the animal is then humanely killed by a Schedule 1 technique and death confirmed at the end of the process.

For relatively smaller samples of blood from larger animals e.g. sheep and cattle, the animals will be subjected to simple venepuncture with the use of local anaesthetic where appropriate. This is a recognised mild procedure, transient in nature with a similar experience for the animals to that experienced by human blood donors.
Why can’t you use animals that are less sentient?

Adult animals are used for blood sampling as their larger body size correlates to a larger blood volume, therefore fewer animals are required to obtain the required sample size. Where large blood samples are required from rodent, the procedure will be conducted under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The welfare costs to animals will be minimised by the provision of group housing with environmental enrichment, bedding and or nesting material as standard. In the case of cattle and sheep, access to secure grazing will be provided during appropriate ambient conditions. Animals will be handled and restrained with the minimal effective restraint. In the case of mice, the refined mouse handling technique will be used so that, as with rats, the use of the tail for handling and restraint will be minimised. For cattle and sheep, animals will be fully acclimatised and trained in the handling and restraint procedures, with

purposely designed race and crush available for use with the cattle.

The potential for pain and inadvertent infection will be minimised by use of single use sterile needles of the appropriate size for the procedure. Local or general anaesthesia will be applied, where appropriate, under the direction of the NVS. Injection sites will be clipped or shaved and cleaned ahead of injection to minimise the risk of contaminated injections and to allow regular monitoring of the injection site after the procedure. Veterinary intervention will be promptly sought as required and appropriate humane endpoints applied in the unlikely event that adverse effects develop and cannot be controlled. Full records of procedures undertaken, daily monitoring and veterinary requests will be maintained using both hard copy and a proven electronic facility management software.

All licensees and animal care staff will be trained, supervised and signed off as competent for the procedures they will undertake.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA and NC3Rs best practice guidelines will be followed in respect to refining blood sampling and injection technique. The NC3Rs grimace scales will be used to monitor rodents and sheep for potential signs of distress. Home Office and FELASA severity information will be used to ensure that the actual severity experienced by the animals can be recorded and limits within this licence adhered to. Body condition score charts specific to the species will be used.

Home Office Code of Practice will be used to ensure animal care and housing is appropriate. This guidance will be used in conjunction with the advice available in the NC3Rs Resource Hub for both housing and handling of animals.

The NC3RS Procedures with Care resource will inform personal licensees of refinements in the conduct of the minor procedures undertaken in this licence.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stay up to date with advances in the 3R's by working closely with NC3Rs programme managers, by maintaining links with the NC3Rs and reading the information cascaded via newsletters from this organisation. I will regularly visit the NC3Rs website and attend appropriate meetings and webinars
e.g. the recent webinar relating to the Scientific validity and EURLECVAM recommendations for the replacements for animal derived antibodies.

I will also receive electronic and hardcopy journals relating to laboratory animal science via my membership of LASA and the IAT and links to FRAME.

In addition, I will look at incorporating and implementing advances via the AWERB and NC3Rs programme manager with licence holders and academic users of this project licence. I have also made contact with a research colleague involved with the development of non-animal derived antibodies with a view to expanding this initiative across the establishment where appropriate.

173. Single/polymicrobial infection models and host responses for novel antimicrobial 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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Infection, antimicrobial, prevention, therapy, biomaterial

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is the development of novel anti-infective strategies for the prevention and control of microbial infectious agents in single or poly-microbial infections. Using rodent infection models, we will identify new antimicrobial and/or immune regulatory therapeutic approaches in the fight against microbial infections. New biomaterials for the prevention of infection on clinical devices such as catheters or new chemotherapeutic agents for the treatment of infections which are resistant to antibiotics in use today. We will use whole animal imaging to reduce the number of animals we need to use as we can observe and assess the changes within the same animal in a non-invasive manner. The primary objective is to identify and improve the translation of anti-infective strategies from laboratory to 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?
Importance:

The United Nations has identified a “global health emergency” with the increase in antimicrobial resistance throughout the world and the failure to develop novel approaches for the treatment and prevention of infection. The organisation for economic co-operation and development has predicted 2.4 million people in Europe/North America and Australia will die from infections with resistant microorganisms in the next 30 yrs. at a cost of $3.5 billion per year. Low to middle income countries already have high antibiotic resistance rates with 40-60% of infections in Brazil/Indonesia/Russia caused by multi-resistant microorganisms. The economic costs of getting a new antibiotic into the clinic and the withdrawal of most major pharma from antibiotic discovery and development compounds has compounded the problem of multi-antibiotic resistance.

In parallel, a joint strategic agenda has been initiated in Europe https://www.jpiamr.eu/ with a remit which includes the development of novel antimicrobial strategies including the discovery of new targets; the development of novel drugs for these targets and novel diagnostic tools for diagnosing infection to facilitate the appropriate usage of the new compounds.

At present treatment relies on the use of broad-spectrum antibiotics but the increasing development and spread of antibiotic resistance and the lack of novel approaches translating to clinic, this is moving us into an world in which we will not be able to treat diseases which we can treat today.

To be able to move through this bottle neck in anti-infective scientific discovery, development and to provide strategies for the control and prevention of infection without the selection pressures of bactericidal agent, novel treatments or improvements in diagnosis are urgently required.

What outputs do you think you will see at the end of this project?

Outputs expected from this project include.

New targets for the development of antimicrobial therapies. Prevention of infection in clinical indwelling devices Treatment of antibiotic resistant microbials

- The improvement of infection modelling and the development of a more clinically relevant poly- microbial infection model.
- Reduction of the bottle neck in the development and improve the translation of the approach from drug development into use.

Publications for dissemination to the wider community.

- For the improvement of animal infection modelling and the use of novel imaging approaches for multiplexing responses within the same animal, reducing the number of animals required and refining the research.
- Identification and validation of novel targets for the treatment of infections.

Products and therapies for treatment or prevention of an infection within a clinical and community setting.

Who or what will benefit from these outputs, and how?

This work will generate both basic and translational benefits

Early Benefits include;

Basic scientific outcome for the benefit of the scientific community.

A greater understanding of infection-host interactions and the identification of new targets for the development of novel chemotherapy development, identification of biomarkers in a more clinically relevant setting, thereby shortening the drug development timeframe.

A greater understanding of the disease site accessibility for treatment. Improving the range of treatments approaches for targeted drug delivery by using data via tracking of target expression/delivery using in vivo imaging. Such benefits are likely to be realised within 5-10 years as we are carrying out our own research and working with collaborators to identify such targets.

A greater understanding of the host and pathogen responses, which will be able to translate into more clinically relevant 3D and 2D cell in vitro models, realised within 2-5 years. Improving the in vitro validation approaches and reducing the total numbers of animals needed for evaluation.

Reduction of animal numbers required for the in vivo validation of infection, and chemotherapeutic efficacy. The refinement of the animal model by the improvement of validation of targeting to the infection, using imaging to enable appropriate timely therapeutic application with novel antimicrobial compounds, reducing the failure to translate into the clinical arena.

Later benefits, for the clinical and community; likely within the 10-20 years.

Improvement of the translation into clinical drug trials by using appropriate preclinical models to screen applicability. At present there is a lack of translation due to the inappropriate approach used preclinically in vivo prior to human trials.

Improvement of understanding of treatment approaches, to enable selection for the most appropriate target and the appropriate treatment.

The development and validation of a novel polymicrobial model, improving infection modelling and drug validation studies.

The progression of the compounds into the clinical trial arena.

Additional benefits.

The use of publication in academic journals and presentation at conference/meetings will enable other researchers to use the models/data to develop new treatments/diagnostic assays.

The dissemination of best practice within the 3R's arena, of improved techniques and approaches.

We will be able to provide modelling expertise to non-collaborative partners with their own drug development programme.

These outputs will further enhance the impact of our work, increasing its extent and the probability of the benefits being realised within the timescales above.

How will you look to maximise the outputs of this work?

We aim to maximize outputs for this work by:

Continuing to work with my already active collaborators which are associated with the previous PPL.

This will enable the dissemination of new knowledge and approaches to the wider research community, not limiting to successful publication in peer reviewed journals but acknowledging within these publications targets which were not relevant for further assessment.

Dissemination of refined approaches to a larger research community, by timely publications and international meetings and the development of new collaborations.

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 use of animals is required due to the complex regulation and nature of infectious disease which we cannot replicate in vitro.

We are using adult mice with a mature immune system to lessen the effect from the microbial infection on a host with an immature or gnotobiotic background which would have a more dramatic adverse effect when challenged by an infectious agent and would not enable translation of the work in a clinically relevant model.

Typically, what will be done to an animal used in your project?

In all cases the animals will be routinely anaesthetised for a short duration for imaging, foreign body implantation and microbe administration as we need them to be still.

To model infection on medical devices we are implanting sterile foreign body supports subcutaneously either using a small-bore needle (when using beads) or via a large bore Trocar needle. Analgesia will be administered prior to the use of Trocar needles but not the small-bore hypodermic needle. The foreign body supports will be either sterile throughout the whole experimental timeline so we can ascertain the hosts immune and foreign body response, or they will be deliberately infected with known doses of single or

poly-microbial populations to generate small discrete clinically relevant infections, which will be tracked using whole animal imaging. The microbes we use generate bioluminescence if they are alive. If bioluminescence is present then the infection is active, if there is no light then the infection is not active.

The imaging employed is non-invasive and combined with our experience in both imaging and research of new medicines any potential adverse effects can be managed and minimised.

We are developing new antimicrobial therapies to treat clinically relevant infections. These will be administered to treat the symptoms of disease, so testing the effectiveness of the new antimicrobial. We will use routes that are appropriate for each type of new compounds, if multiple doses are required, a slow drug delivery system will be used to reduce the number of injections.

Using imaging technology, we can reduce the number of animals as disease progression can be monitored within the same animal over time.

Duration of infections will be the minimum required to establish a clinical disease and assess the antimicrobial drug action. Daily imaging, up to a maximum of 14 days, will assess early response and treatment of infection. Loss of bioluminescence, suggesting an inactive infection, will result in a premature study end compared to traditional experimental approaches.

To replicate clinically relevant infections associated with long-term implanted clinical devices such as pacemakers some studies will require microbial infection 30 days after insertion of a the support.

What are the expected impacts and/or adverse effects for the animals during your project?

After implantation of the foreign body supports the incision site will undergo a wound healing response, in mice this is a contraction of the incision site and the formation of a scab over 7 days. An inflammation event may occur which is observed with a change in colour and small swelling of the insertion site.

Infected animals may present with clinical signs of infection which include piloerection, changes in skin colour at microbial infection site, subdued behaviour patterns, more than a transient hunched posture, pallor of eyes, nose, ears and foot pads, body weight loss of less than 20% but typically is 10%. The severity of these adverse effects will be microbial strain dependent; we will use the lowest number of microbes we need to develop a clinically relevant infection.

In the first 72 h of the infection animals are expected to display clinical symptoms associated infection, after which the health status should improve, this is normal, it is the immune reaction to the microbe, like a human cold. The infection site is expected to undergo a typical response, raised swelling and possible scab, this can take up to 7 days as the immune response detects and controls the infection.
100% of the infected animals are expected to show the initial response to the infection, with the use of antimicrobial therapy the infection is expected to be treated and cleared from the animal.

All microbes will be pre-screened typically via in vitro and/or non-vertebrate models prior to administration into mice to screen for virulence. No adverse effects are expected from in vivo imaging acquisition, the anaesthetic events are typically of a short duration.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In our experience given the requirement for anaesthesia and microbial infection 100% of animals are expected to experience up to moderate severity for 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?

Due to the complexity of the infection process and to permit the translation of novel antimicrobials/biomaterials into clinic/veterinary/agriculture arenas we need to demonstrate the efficacy of these compounds/materials. To enable us to achieve the objectives of this project, we need to be able to study the interaction between host/microbes/antimicrobials in a physiologically relevant system. Mice are the lowest neurophysiological sensitivity organism retaining similar physiological characteristics to humans and are well characterised models of infection.

We are not able to completely reproduce the complex interactions and communications that occur between microbial pathogens and their host in in vitro models. For much of the work it is not possible to model the in vivo regulation of the microbial association with the immune system using cell culture systems or the systemic efficacy of novel antimicrobials. This can only be achieved using whole animals or humans; we cannot use humans and therefore we must use animals. Animals will only be used in experiments where it is not possible to perform studies by other means and only after a lot of in silico and in vitro development.

Which non-animal alternatives did you consider for use in this project?

A large proportion of the initial screening involved in the characterisation of the host response and microbial interactions will be carried out in silico, in vitro virulence assays and using non-vertebrate in vivo models. Through this approach we have identified new therapeutic targets, which have enabled the further development of novel antimicrobial compounds.

We typically apply the following approach to the development and testing of novel compounds:

Using computer modelling and data mining in silico we profile compounds via virtual screening of candidate molecules against a diverse range of microbial protein active sites, identification of potential targets and generation of potential pharmacokinetic /pharmacodynamics parameters leading to the identification of biochemical pathways which control microbial virulence.

Once potential target compounds have been identified we then typically assess their efficacy by assessment in the following in vitro models: Determination of the effect of the antimicrobial on planktonic culture growth and viability; microbial attachment on medical device surfaces i.e., catheters/surgical mesh.

Determination of effect of the antimicrobial on changes in virulence gene expression in the microbes.

Determination of any changes in host interaction, haemolytic activity against red blood cells, toxicity, immune cells response, serum activation/inactivation.

Once we have confirmed the activity of the novel antimicrobials in vitro¸ we typically determine the activity in a more complex model system. These include: In vivo in non-vertebrate's wax worms. or Ex vivo pig lung infection models, human and murine tissue derived skin infection models.

Why were they not suitable?

Due to not having a complete immune system, the in vitro tissue and non-vertebrate models do not provide the full complexity of in vivo interactions.

This can only be determined in a living host, with an active innate and acquired immune system, metabolism, and circulation for the bio distribution of antimicrobial chemotherapy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 inoculation doses and duration of infection, immune response and pathology of parental microbial species are therefore already known for two of the microbial organisms we use and will be used as the basis for the development of the other species and poly-microbial infection. This project licence proposal is a progression from work developed under the previous licence; we have developed a novel non-invasive whole animal imaging strategy to allow real time detection of the microbial infection within the same animal.

Which allows us to substantially reduce the number of animals we need to use. An example of this, using traditional animal work a 5-day infection study would use 120 animals as we would need to sacrifice at each time point, by using imaging we have reduced the number of animals we need to 24. Using this approach, we have successfully

translated a concept from in silico, in vitro, in vivo and it is now undergoing clinical trials.

Using the same approach through this project we envisage testing of up to 100 novel compounds/antimicrobial/biomaterials strategies in mice. Initially in year 1, these will include panels of 10-12 lead compounds/antimicrobial/biomaterials which previously identified through in vitro screens, which either inhibit; i) microbial growth, ii) microbial surface attachment, iii) microbial cell-cell communication and/or iv) microbial virulence factor production.

Despite initial in vitro toxicity testing, it is possible that some of these compounds may still cause unacceptable side effects when assessed for acute toxicity in mice and consequently will not be evaluated further at this stage.

Where compounds are well tolerated, these will be assessed for efficacy against the test bacterium in the relevant infection model using a block design with small groups of animals (typically n=2) and a common control group. Based on this data, where appropriate, the study will be repeated or possibly modified (e.g., change of dose or dosing frequency) again using a block design, to confirm the initial results. Compounds/biomaterials initially showing significant activity in mice i.e., clearance of the bacterial infection will then be tested further (e.g. dosing studies, variation in route and frequency of administration) and those with the best spectrum of activity identified for clinical translation.

Total numbers of compounds to be tested in vivo and extent of testing required will be dependent on results obtained so is difficult to predict with precision, but evaluation of up to 100 compounds compounds/antimicrobial/biomaterials, if required is considered feasible.

At present we are testing 60,000 compounds/antimicrobial/biomaterials against proposed virulence targets in vitro.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We utilise the NC3r's Experimental Design Assistant as a basis for our experimental design.

Using small defined validity studies will enable the assessment of the potential de novo antimicrobials use for preventative or treatment of infection. An antimicrobial might be more suitable in preventing colonisation rather than clearing an established infection, the working concentration of the antimicrobial may be changed from in vitro validation data. By using small validity studies, we can check the efficacy of the antimicrobial, virulence markers and novel imaging substrates prior to expanding the study.

Data from validity studies will uncover the potential in vivo efficacy of antimicrobial compounds in prevention and/or treatment of microbial infections. If no changes in bioluminescence or colony forming units occur, then typically the antimicrobial concentration will be adjusted and re-assessed in in vitro screens prior to re-assessment in the in vivo model. Typically, the antimicrobial validity studies will be grouped together, allowing more than one antimicrobial to be assessed and permitting the use of the same control animals for multiple antimicrobials to reduce the number of animals used.

What measures, apart from good experimental design, will you use to optimise the

number of animals you plan to use in your project?

A pilot/validation study will be employed for all new imaging markers, antimicrobials, and microbial virulence assays to demonstrate the validity of undertaking a larger animal cohort experiment. Any adverse clinical symptoms observed will prompt reassessment in vitro of the microbial strain/antimicrobials, prior to continuation of animal work. Experiments will be carried out according to analysis of variance to see if the data meets the assumption of Anova, if this is not possible and statistical analysis carried out using non-parametric tests which are appropriate for small sample size, which do not meet the assumption of parametric tests. The Mann-Whitney U-test will be used to determine differences between sets of independent data from two treatment groups, e.g. bioluminescence levels, and the Kruksal-Wallis test for equality of medians will be used to compare the results from multiple groups. Replicates of experiment typically 3×.

The studies are designed to be interactive and provide information to minimise the number of animals utilised.

Tissue bank, containing frozen and fixed immune organs, infection site and other organs. For retrospective validation of possible new targets without having to use new mice. The frozen infection sites permit the assessment of phenotypic changes in the microbial populations due to selection pressure of the host and the treatment.

GM mice and microbes expressing multiple markers for imaging, multiplexing, and reduce the number of animals required.

The output from each animal model has been designed to provide a diverse tool for the complex host/microbial/antimicrobial response.

Using imaging approaches, we can reduce the number of animals required as we are looking at changes within the same animal over time, increasing the scientific output and reducing the variation between responses on a spatial time course. Using optical and/or nuclear imaging we can obtain the following data in real time:

Location of both metabolically active and senescent bacteria within the infection site.

The validation of microbial virulence targets using the microbial genetic mutants, including the analysis changes of the host immune response to these microbes.

Quantitative changes in microbial virulence gene expression by detection of bioluminescent light produced from the lux operon or other markers controlled by virulence gene promoters; this will provide information for the targeted timely application of the appropriate antimicrobial.

The antimicrobial efficacy of test compounds and microbial presence in the infection site by following changes within a single animal, including the assessment of possible re-colonisation of the infection site after antimicrobial withdraw. For most of the animal studies, inbred strains will be used to reduce the number of animals required per group to achieve statistically significant results.

Data concerning the maximum number of parameters possible will be obtained from each experiment to maximise the information obtained from each experiment and prevent unnecessary repetition.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use mice for infection model experiments to accurately model the disease which is translatable to that of human infection. Mice have the lowest neuro-physiological sensitivity yet retaining similar physical characteristics to humans and are also well- characterised models for research infection modelling.

The types of infection model we are proposing enable us to look at accessibility, response to antimicrobials and validation using non-invasive imaging approaches. Unlike other infection models with traditional sequential endpoints, we use whole animal non-invasive imaging to follow within the same animal, utilising the least inoculation dose of microbes, within a discrete surface localised infection site.

General infection refinements.

Fitting in with the circadian rhythm of the nocturnal mouse. We have noted that we get reduction in host infection clinical phenotype, by starting the experiment to fit in with the mice’s natural nocturnal cycle, not forcing the mouse to fit in with our working day.

As we are tracking light produced by the live bacteria we can use this to end an experiment early. If the treatment works well and the bacteria are not alive after 2 days of treatment, there is not light, this allows to end and experiment early. So reduction and refining the experience for the animal.

Drug delivery

To refine and reduce the impact of therapeutic drug delivery, where appropriate, we will use an implanted subcutaneous drug delivery system i.e. osmotic mini pump or slow release bio-gel to allow administration of compounds to reduce the number of procedures for each animal.

Foreign body response and infection.

We use analgesia when we implant the clinically relevant foreign body supports are sterilely implanted to remove any pain related response. The length of foreign body will be the smallest required for establishment of microbial infection or host foreign body response, with the least impact on the host.
Microbes are administered under anaesthesia using an insulin needle to reduce stress to the animal. The use of a foreign body allows the reduction of the microbial inoculum CFU compared to the literature, reducing the early response of the host to an excessive microbial load and the overall health impact on the animal.

Poly-microbial infections

The infection sites within the human and mouse host are naturally not made of a single species of microbes, we will extend the scope of the work to cover these important infection scenarios. Initial we will use Pseudomonas aeruginosa, Staphylococcus aureus for foreign body models, as these have been shown to be clinically relevant and will look at the interaction of the host commensal microbiome.

Transgenic animals

Only animal strains whose transgenic status has limited health impact on the uninfected animal will be considered for this work. The use of strains lacking or over expressing components of the immune system will permit the assessment of the host response to a specific virulence pathway, without the need to use additional compounds to remove immune components, i.e. the use of high concentrations of endotoxin or cyclophosphamide both of which have high detrimental health impact on the mouse.

Imaging refinement

Whole animal non invasive imaging technology permits the detection and the analysis of a response within the same animal over time. We can look to see where the bacteria are, how many are there and also we can ask more than one question at a time, for example is the immune system responding. We are developing approaches to enable multiple biomarkers to be analysed simultaneously, improving sensitivity so reducing the requirement for duplication of animal experiments and refining the experimental approach.

We are undertaking the development of approaches to enable more sensitive detection of microbial number by imaging; again, this will enable us to reduce the initial microbial inoculums required to establish a detectable infection. Real-time imaging modalities also permit the early termination of models in which the infection has been successfully treated, reducing the length of the experiment and the impact on the mouse.

Sampling methods that minimise animal suffering included the following:

Micro blood sampling from tail and urine collection from the cage floor.

Anaesthesia

To carry out whole animal imaging we need to use anaesthesia to immobilise the animal for the imaging acquisition, this is deemed less stressful than restraints. The animals are maintained in a warm environment to maintain core body temperature and the anaesthetic events are of short duration, the animal is recovered with adlib food and water and the animals have access to mash. For longer anaesthesia events the animals are recovered in their cage on a warming mat.

Pilot studies

Pilot studies and small validation studies will be employed when using new antimicrobials/microbial strains/species and new improved imaging approaches to ensure there are no unexpected adverse effects associated with the new models.

Why can't you use animals that are less sentient?

We do use animals which are less sentient in the form of non-vertebrate models, Galleria mellonella larvae. But we need the dynamic complexity of the mammalian host for a clinically relevant infection and host interaction which is not available within this non-vertebrate host.

We use fully immune matured mice to enable an easier translation into clinic and less adverse effects to the mouse. Using mice with an immature immune system will give an enhanced adverse response to the mouse and as clinical trials typically are not carried out on children, we feel that ethically it is more responsible to validate using an adult murine mature immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The experimental protocols at present have many refinements which have been developed through work undertaken under the previous project licence.

Monitoring is always a constant refinement, we monitor in relation to the health status of the mouse, increasing if the clinical signs change.

With the improved sensitivity of imaging approaches, we can reduce the number of microbes we can track by in vivo imaging approaches and the multiplexing of host response, constantly improving the information and minimising the health impact on the animal.

The length of foreign body will be the smallest required for establishment of infection with a microbial pathogen, which are administered under anaesthesia using an insulin needle to reduce stress to the animal.

Pilot studies and small validation studies will be employed when using new antimicrobials and microbial strains/species to ensure there are no unexpected adverse effects associated with the new models. Where necessary and as advised by the NVS analgesia will be administered to minimise pain from the infection model.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All procedures will be carried out with best practice where appropriate excites current published best practice exists and from talking to colleagues if not published.

Principles of the 3R's – National Centre for the Replacement, Refinement & Reduction of Animals in Research

Guidelines to be adhered to include.

- ARRIVE
- Responsibility in the use of animals in bioscience research FELASA guidelines and recommendations
- How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This project is always dynamically looking to improve the validation of host response, microbial antimicrobial response, and imaging approaches. To keep informed on the advances within the area of 3R’s we will consult with the NC3R's programme manager the NC3R’s website, LASA guidelines, conferences, and literature reviews.

174. Understanding the mechanisms underlying progression of Chronic Kidney 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

Kidney disease, Fibrosis, Therapeutic targets

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To better understand how kidneys respond when injured and to identify new ways to treat injured kidneys to prevent long term 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?

The Institute for Health Metrics and Evaluation’s Global Burden of Disease Study 2017 highlighted that chronic kidney disease (CKD) is predicted to rise from the sixteenth (in 2016) to the fifth most common cause of death worldwide by 2040, overtaking diabetes. Although this rapid rise is partly driven by population growth and ageing, there is currently

no disease-modifying drug that preserves kidney function in patients with CKD, which will allow us to impact on this predicted epidemic. There is therefore a pressing need to better understand the complex mechanisms that lead to chronic renal dysfunction so that we may develop drugs that have the potential to slow or prevent the progression of this disease and to save lives.

What outputs do you think you will see at the end of this project?

By the end of this project we hope to have increased our understanding of why kidneys fail and how we can intervene to stop or slow this process. We intend to publish our results in high impact, peer- reviewed journals to allow for maximum dissemination and to share our findings with industry and influence their choices when investing in new potential therapeutic agents.

Who or what will benefit from these outputs, and how?

The beneficiaries will include:

- Patients with kidney disease who may have access to new clinical trials or drugs proven to have efficacy in treating their disease
- Other researchers who can build upon the work we have done in refining our animal models
- Industrial partners with whom we can collaborate to translate our results to the clinical arena.

How will you look to maximise the outputs of this work?

We already have a significant collaboration established with other groups working on renal fibrosis which allows us to work closely together and build upon each other's findings. We will endeavour to publish all of our results, regardless of whether they are positive or negative, but if unable to publish negative data we will share these with collaborators to ensure similar experiments are not needlessly conducted.

Species and numbers of animals expected to be used

- Mice: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using adult mice to mimic acute and chronic kidney disease in humans. We have chosen mice as the least sentient mammal that responds to kidney injury in a similar way to humans. Chronic kidney disease is more common with increasing age and in men so we have chosen to use adult male mice to best reflect the patients we aim to help with the results of this work.

Typically, what will be done to an animal used in your project?

Most mice will undergo one or more injections of a substance that is known to be toxic to the kidneys. This will induce an acute kidney injury which will result in a transient rise in the levels of waste products in the blood and a small drop in body weight. This will resolve over 3-5 days. Depending on the strain of mouse and the toxin used, this may be repeated between one and five more times. After this, animals will be maintained for up to 5 months to allow a scarring process to develop in the kidneys which leads to chronic kidney disease. Some of these animals will receive compounds (orally or via injection) which are being studied as potential treatments to prevent kidney scarring.

Some mice (<10%) will have one kidney surgically removed before receiving the toxin injections. This will accelerate the scarring process and reduce the length of time the experiments run for.

Some animals may undergo total body irradiation and bone marrow transplant prior to receiving the injections to allow us to study which immune cells travel to the kidney and contribute to the scarring process.

Following the acute kidney injury, some animals will be anaesthetised and imaged between one and three times to better understand the processes occurring in their bodies without the need to kill them.

What are the expected impacts and/or adverse effects for the animals during your project?

During the acute kidney injury phase, animals will develop a rise in waste products in the blood but this is not expected to be severe enough to cause suffering. More severe kidney damage may occur in some animals and may lead to loss of appetite, weight loss, and dehydration or fluid gain. This is expected to occur in < 10% of animals.

Animals undergoing surgery may experience post-operative pain which will be treated with pain killers and is expected to last for <12 hours. A few animals may have a breakdown in their wound which will need to be stitched one more time with the use of local anaesthetic.

Animals undergoing bone marrow transplantation may experience transient diarrhoea, shortness of breath and weight loss. Any animals suffering with severe adverse effects, or those lasting more than 24 hours (shortness of breath) or 48 hours (diarrhoea) 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)?

The severity of the nephrotoxic protocol is moderate but we anticipate less than 10% of animals will reach this 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 kidney is a very complex organ formed of multiple different cell types organised in a particular fashion such that function is dependent on structure. The development of renal fibrosis is a complex process involving the interplay of multiple different cell types at different stages of development. As a result, there is no in vitro model that can adequately mimic these interactions, let alone mimic the function of a normal kidney. As a result, in vivo models are required to understand the development of renal fibrosis, and to assess potential treatments. There is, however, a significant role for in vitro systems for investigating and understanding the cellular processes involved and screening potential treatments. Those insights generated from these in vitro experiments, and candidate treatments demonstrating promise, will then be taken forward to animal studies.

Which non-animal alternatives did you consider for use in this project?

Primary cell cultures and immortalised cell lines of various cell types (including renal tubular cells, pericytes and monocytes) have all been considered as part of the in vitro elements of these studies. Recent developments in the field of regenerative medicine have led to the development of renal organoids; simplified and miniaturised subunits of a normal kidney. However, these renal organoids currently lack a functional blood supply and all of the normal cells found in a kidney.

Why were they not suitable?

Both of these potential non-animal alternatives lack the complexity of the normal kidney, and the large number of resident and blood-derived infiltrating cells that interact during inflammation and fibrosis and are therefore unable to adequately model this complex disease process involving interplay of multiple elements.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 my previous Return of Procedures forms to calculate how many animals I have used over the last 5 years and have extrapolated forward based on a similar degree of productivity. I have then reduced this number by 10% to account for improved access to human tissue and primary cells that we are planning to introduce. This is a conservative estimate and we hope to reduce the use of animals by more than this.

What steps did you take during the experimental design phase to reduce the

number of animals being used in this project?

I have taken extensive advice, in the past from statistical colleagues. The basic design of in vivo experiments has not altered over the years; all involve comparison of a control group with an experimental group with a specific biological read-out. Use of 6-7 animals per group, in each experiment, will enable the differentiation of a 20% difference in read-out between groups at 90% power and 5% significance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We plan to collect all tissue, serum and cells from every animal used in each experiment, and store appropriately, in a local tissue bank in our laboratory, to avoid having to repeat experiments to examine novel aspects of the model - we can instead revert to stored tissue to ask exploratory questions, only having to use new animals if our exploratory findings are positive. We also have access to animal tissue from experiments previously carried out by our collaborators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We plan to use models that mimic human disease but which confer the minimum degree of suffering to the animals. These include injection of a substance called aristolochic acid, which is found in some Chinese herbal medicines and is known to cause significant chronic kidney disease across the globe. Although high doses of this substance can lead to severe acute kidney injury and death, we have significantly modified this protocol by reducing the dose and increase the administration frequency.
This causes a milder acute injury but a more rapid progression of (minimally symptomatic) chronic disease. This allows us to study the underlying mechanisms that lead to chronic disease over a shorter time period, with less suffering to the animals.

Why can’t you use animals that are less sentient?

The renal system in mammals is very complex and not adequately modelled in less sentient animals. The process of scarring is driven by the interactions of multiple systems including the immune system, metabolism and wound healing which have evolved to a high degree in mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are constantly thinking of new ways to minimise harm in our models. The animals are frequently monitored for signs of suffering and we ensure an adequate amount of

analgesia is used when needed. We also minimise handling of the animals and house them in groups when at all possible to allow socialising. If animals are purchased from a commercial breeder they are allowed to acclimatise after delivery for 1 week before being entered in to a protocol to avoid adding one stress to another. If an animal reaches an humane endpoint, we ensure the animal is killed quickly and humanely.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We aim to report our work using to the latest ARRIVE guidelines (2.0). Following these guidelines will ensure our experiments 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 have put the 3Rs are a standing agenda item at our collaborator meetings. We share best practice in experimental design and keep up to date with the relevant literature and information from the NC3R website.

175. Developing targeted immunotherapy for tissue regeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (ii) 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

Barrier Tissue, Tissue Resident Immune Cells, Homeostasis, Inflammatory diseases, Cancer

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?

By exploring the functions, molecular mechanisms and induction methods of immune cells residing in barrier tissues, naming skin, lung and oral cavity, we aim to develop an immunotherapy to modulate the abundance and/or functions of these tissue resident immune 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?

Barrier tissues, such as skin, lung and oral cavity, are our first line of defence against any exogenous threats, such as pathogens, radiation, toxins and physical trauma. The

dysfunction of these organs can lower one's quality of life significantly, and in some extreme cases death. In skin, 54% of the UK population suffer from some form of skin disease each year, costing the NHS in the region of £723 million per year, based on a report conducted in 2016 (ABPI DI report, 2018). According to a survey from the British Association of Dermatologists in 2011, 85% of patients suffer psychological stress due to their skin conditions. Lung disorders are estimated to affect 1 in 5 of the UK population, contributing to 1 in 5 of all deaths and costing the NHS over £11 billion a year (British Lung Foundation Report 2016). For example, the outcome from idiopathic pulmonary fibrosis (IPF, a chronic and progressive scarring of the lungs) is extremely poor, with a mortality rate of 50% at the point of diagnosis and 80% within three months of an acute exacerbation. Furthermore, poor oral health can lead to long term pain, discomfort but also negatively affect one's confidence, and costing the NHS around £3.4 billion per year (NHS website, 2019). One example is periodontitis, a form of gum disease characterised by the accumulation of oral bacteria, resulting in chronic gingival inflammation, and bone loss. This disease is prevalent in 50% of the population worldwide, with 10% affected by its severe form. Periodontitis is also closely linked oral squamous cell carcinoma, the most common oral cancer in UK accounting for 9 out of 10 cases.

Yet, current therapies for these barrier tissue diseases remain largely generic, and clinical strategies to specifically target barrier tissue pathologies remain scarce. For example, treatment of skin diseases relies on generic topical treatment (such as steroid creams) or systemic therapies (including many immunosuppressive drugs). The use of such systemic approaches can bring upon many adverse side effects. Perhaps the best example is the use of Adalimumab (a TNF inhibitor) to treat the human skin inflammatory disorder, psoriasis, that may impart severe secondary infections, headache and nausea, given its global immunosuppressive effect (NHS website, 2018). Similarly, treatment of idiopathic pulmonary fibrosis in lung (IPF) currently relies solely on lung transplantation. It is fraught with shortage of donors, post transplant rejections and over-use of immunosuppressant drugs. In the case of oral diseases (such as periodontitis disease), prevention remains a major strategy. Grafting of soft tissues or bones have been used as common strategies to help reduce further gum recession and to prevent tooth loss. Although guided bone regeneration or proteins stimulating bone and gum regrowth, these are difficult to target the root of the causes of periodontitis.

Tissue pathologies of the skin, lung, and oral cavity are initiated, maintained, and repaired by both epithelial stem cells and immune cells. An intimate co-operation between these cell types maintains tissue homeostasis in the steady state and/or promotes regeneration in response to injury. Importantly, immune cells that directly impact tissue biology are, in fact, resident within the tissue itself. The role of tissue resident immune cells (TRICS) has been woefully ignored, as has their interactions with epithelial and stem cells. Our project is unique and clinically relevant given that our major focus is the analysis of TRICs. Utilising mouse models that closely recapitulate human tissue dysfunctions, we propose to spatially, temporally and functionally map the role of immune cell-epithelial stem cell interactions throughout the spectrum of tissue pathogenesis. There are indeed specific immune cell subsets that have been implicated in this dynamic, including but not limited to, regulatory T cells (Tregs), tissue dendritic cells (DCs), and macrophages. Our unbiased and hypothesis-driven approach aims to dissect the functional contribution of these cell types and to identify the major pathways these cell types utilise during disease progression. Our discoveries of both tissue-restricted and specific cell type expressed factors will serve as targets for the development of biological therapies to treat human tissue regenerative diseases of skin, lung, and oral cavity.

Recently, skin Tregs have been shown to facilitate epithelial stem cell function and thus modulate tissue regeneration. The exact molecular mechanisms of how Tregs or other TRICs influence epithelial stem cells is unknown. Our research will explore immune cells residing in skin, lung, and oral barrier tissues under 3 shared aims: (i) understanding how TRICs can contribute to barrier tissue homeostasis; (ii) how TRIC dysregulation may lead to tissue regenerative disorders, fibrosis, and cancer; and (iii) how to artificially manipulate TRICs. Mice are an excellent model to study the highly complex networks that TRICs have established with the local environment in vivo, and subsequently indicate how they may contribute to barrier homeostasis in man. Importantly, by dissecting TRIC-mediated pro-regenerative mechanisms in mouse, we aim to develop better therapies in man. Developing a localised approach to target a specific immune cell population will be critical to improve treatment efficacy.

What outputs do you think you will see at the end of this project?

Understanding which immune cells are involved in the regeneration of barrier tissues, and key signalling pathway employed.

Delineating the mechanism of TRICs in barrier tissue regeneration. Publication(s) in high impact peer reviewed journal, and potential molecules for clinical use.

Establishment and sharing of (a) protocols for characterising skin, lung, and oral cavity resident immune cells and proliferating epithelial cells in normal and diseased human tissues (b) robust, atraumatic in vivo models of normal regeneration and aberrant regeneration and robust lung, skin and oral oral regeneration end points (c) robust human ex vivo skin and lung models, with physiologically intact matrix, endothelial and epithelium and robust epithelial regeneration readouts

Deeper understanding of the role immune cells in fibrosis, inflammatory disease and cancer manifestation in skin, lung and oral cavity

Who or what will benefit from these outputs, and how?

This research would provide the basis for highest calibre tissue immunology expertise to the UK to address the major immune system challenges limiting sustainable delivery of stem cell and/or immunotherapies and endogenous regeneration approaches at scale. The identification of the molecular mechanisms facilitating Treg-mediated stem cell activities, and their upstream regulation, will pave the way to developing the clinical application to specifically target tissue Tregs, and subsequentially enhance regeneration post damage as well as other tissue-Treg related inflammatory diseases/cancer.

The academic impact of advancements in the field of tissue resident Tregs, other immune cells and tissue epithelia in regenerative medicine is high. Thus, we envisage significant local, national and international interest in our results. A successful project is also likely to benefit:

Short term: Researchers within the fields of skin, lung and oral cavity stem cells and immunology. Many cellular interactions between immune cells and stem cells are defined by common regulatory pathways that are likely to be relevant to other immunologically active tissue sites. Our work has the potential to address gaps in this field for example - do tissue resident immune cells (TRICs) have a role in regeneration, which immune cells in each barrier tissues, how, and whether these immune cells from different barrier tissue

resemble each other. Our work will address how barrier TRICs may mediate homeostasis. Importantly, we aim to dissect how they may contribute to injurious repair of barrier tissues. In particularly, little is known about the potential link of lung and oral tissue resident immune cells with local progenitors during injury. Thus, we seek to understand if these barrier tissue specific immune populations can facilitate in resident stem cell activity, as previously observed for skin Treg mediated hair regeneration and wound healing. Another major question is understanding whether the same or similar immune cell populations are found in human lungs and oral tissues. There are also several discovery studies (such as the single cell RNA sequencing studies of injury, and normal vs aberrant regenerating tissues and the human immune maps of barrier sites from human clinical samples obtained in the lab) which could potentially change the way we view immune responses in our barrier tissue of interest, as well as other organs. Finally, after surveying the immune cells in each of these three barrier tissues (skin, lung and oral cavity), we can then compare their functions, molecular mechanisms and their regulation, and obtain a complete picture to understand if there are components (phenotypically and functionally) shared among these resident immune cells.

Long term: Biological therapeutics and patients. Knowledge of the cellular mechanisms employed by barrier TRICs (including Tregs) in regulating stem cell differentiation during normal tissue maintenance and post injury could lead to the development of novel chemical inhibitors or activators and specific antibody mediated approaches. In this regard, the long-term primary beneficiaries are the human disorders, alopecia areata (autoimmune-triggered hair loss), melanoma, idiopathic pulmonary fibrosis, lung cancer, periodontitis and oral cancer - patients, clinicians and scientists.

Current therapies of alopecia areata is topical application of corticosteroid, anthralin cream or minoxidil, which are not effective on all patients, nor it is possible to prevent new hair loss. Research focusing on skin Treg function could provide an endogenous approach to resolve hair loss symptoms.

The outcome from idiopathic pulmonary fibrosis (IPF) is extremely poor with no current therapy - patients have a mortality rate of 50% at the point of diagnosis and 80% within three months of an acute exacerbation. Lung transplantation is fraught with shortage of donors, post-transplant rejections and immunosuppressant use. This research could pave the way for the use of endogenous alveolar regeneration in end stage disease in place of, or bridging to lung transplantation.

The onset of periodontitis usually develops if gingivitis is not appropriately treated. Periodontitis can be treated with the use of antibiotics, professional scaling, root planning and cleaning. In its severe form, periodontitis can lead to a release of bacterial and proinflammatory mediators into the blood, causing systemic health complications and puts a strain on the immune system. Invasive measures include gum pocket reduction surgery, soft tissue grafts, bone grafting and guided tissue regeneration with the use of biomaterials. Our study aims to promote oral tissue regeneration involving less invasive/ surgical procedures, by investigating whether endogenous immune cells can support oral epithelial regeneration, as a prospective therapy.

Despite advances in surgery and radiotherapy in treating oral squamous cell carcinoma, the mortality rate remains high. Oral epithelial cell markers have been found to be expressed on cancer stem cells, which contribute to tumour progression, and may be resistant to radiotherapy and chemotherapy. Our project also aims to elucidate role of TRICs in the directing oral stem cells towards either a tumorigenic or a pro-regenerative

phenotype.

How will you look to maximise the outputs of this work?

Both our projects investigating skin and oral cavity resident immune cells are conducted under the close collaboration with other experienced research labs, who have developed multiple sophisticated stem cell murine models (such as inducible transgene knockout, inducible stem cell tracing models, skin injury, melanoma and oral cancer models) but also essential techniques for the success of this projects (including live in vivo imaging, immunohistochemistry).

Furthermore, our project on lung resident immune cells is conducted with the joint effort of three UK institutions. Our collaborators encompass both basic scientists and physicians-scientists with human tissue access and are all leading experts in the field of tissue resident immune cell mapping using multi-dimensional histological techniques, lung fibrosis, and development of patient interventions.
Another collaborator is a respiratory physician-scientist with decades of experience working with mouse models of lung dysfunctions and will serve as our main contact for discussion and sharing of data.

To conclude, we seek to maximise the output of our project by collaborating with a wide range of experts in stem cell biology, molecular biology, biomaterials, and bioinformatics. We strongly believe that by exploring our research from the unique tissue focused perspective. We propose in this application, we can obtain a much more accurate picture of why immune cell-stem cell interactions are biologically significant, and how we can manipulate this axis to both prevent the manifestation and treat the pathology of a wide range of barrier tissue regenerative disorders of man. The main forms of our outputs will be through publishing in high impact journals and presenting these results in national and international conferences.

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.

Mice are one of the best studied mammalian models. Genetically, mice share 85% of their genome with human, which makes it biologically relevant to study human diseases. Mice are well-established experimental models, with a huge range of molecular tools already developed to manipulate their genome precisely, or even introduce mutations specific to human diseases. Additionally, laboratory mice can be inbred to yield genetical identical strains, minimising background genetic variations between individuals and maximising the credibility of experimental results. Furthermore, in comparison to humans, mice can reach adulthood within 6 weeks postnatally, reproduce quickly and provide large litters. This allows us to perform our experiments in a timely manner and with a high degree of study them quickly and with statistical confidence.

For skin: The choice of the life stage is due to the mouse hair follicle (HF) cycle. In mice, throughout life, HFs actively cycle, self-renew, and generate new hair shafts through a repeated cycle of shedding, quiescent and growth phases. For C57BL/6 mice, their HFs begin to form immediately after birth, but only enter their first quiescent phase at 3 weeks of age, and growth phase at 4 weeks Importantly, the first two HFs cycles (1st cycle: 3 to 6 weeks postnatal (juvenile), 2nd cycle" 7 to 14 weeks postnatal (adult)) are highly synchronised in C57BL/6 mice, making the first two cycles perfect model to study hair growth. Skin Tregs have been shown to mediate this HFs transition from quiescent to growth phase. Hence, it is critical we have a model capturing the transition. However, since after the 2nd HF cycle, the hair cycle is no longer synchronised on the dorsal(back) skin. Certain regions of the mouse dorsum may enter the quiescent phase, while others may not. It therefore becomes difficult to study the dynamics of skin resident immune cells with hair regeneration. Thus, we will limit the age of our samples to juvenile and adult mice only.

For lung: It is established that idiopathic pulmonary fibrosis (IPF) can occur in adults, therefore, in order to truly reflect the injury and repair as seen in human adulthood we will be using juvenile and adult mice at both healthy and injured state. To the best of our knowledge, bleomycin instillation is usually carried out on adult mice. As well as studying the involvement of TRICs (including tissue-resident Tregs) in the regenerating the alveolar niche during IPF, we also aim to investigate whether the aforementioned cells influence developmental stages for alveologenesis from neonatal to adulthood at a steady state. Conversely, if a particular condition/ environment produced by the TRICs is identified during alveologenesis at a steady state, these 'conditions' may be adopted as a therapy to promoting alveolar regeneration in a fibrotic setting. The final stage of lung development is defined as the alveolar stage or alveolarization, which occurs at postnatal stage P5-P30 in mouse and the gas exchange surface is fully formed and functional Hence, mice at stages P5-P30 will be of interest in this project.

For oral tissue: We would also like to investigate immune cell and epithelial stem cell interactions in the oral cavity at different ages of mice such as neonatal, juvenile and adult. It is established that in tissues, including lung and skin, the neonatal immune system is highly distinct from adult where this difference contributes to the rate of repair after injury. This is currently unknown in the oral cavity, therefore we aim to perform 4NQO treatment in drinking water and oral wounding for neonatal, juvenile and adult mice, whereas ligature application will be used strictly for juvenile and adult mice to assess immune and epithelial cell dynamics during states of injury/ repair.

Typically, what will be done to an animal used in your project?

A typical experiment to study tissue homeostasis and repair would involve comparison of the skin/lung/oral cavity of genetically altered and littermate control mice without perturbing tissue function, with triplicate mice per condition. To evaluate gene expression the mice could be live in vivo imaged, and prior to killing they may be labelled with EdU to allow determination of the proportion of S phase cells. Induction with tamoxifen and diphtheria toxin to activate/repress genes of interest will normally be administrated every other day. Of note, the concentration and injection interval of tamoxifen or diphtheria toxin will be determined in trial runs before any experiments. All inducing agents may be administrated in parallel to the challenge of barrier tissues, depending on whether there is promoter leakage and inability to delete the majority (i.e. 80%) of target genes.

To challenge skin function, mice would either undergo tape stripping or hair plucking or

wounding or UV irradiation, or injection of cells, but it is very unlikely that more than one of these procedures would be carried out on the same mouse.

For bleomycin injury, mice will undergo anaesthesia induction and will receive one or two infusions of bleomycin via intra-tracheal or intra-oral cavity route. Mice may be bled via tail vein needle prick at specific time-points until 6 weeks post instillation. Mice may be subjected to further inducing agent or drug/antibodies treatments by oral gavage, subcutaneously, intraperitoneally, intradermally, intra -nasal, intra-tracheal or intravenously. These will be administered on one or two occasions up to 21 days post bleomycin instillation. These mice may be subjected to live in vivo imaging under anaesthesia post- instillation.

For ligature application, mice will be maintained under general anaesthesia (administered intraperitoneally) before the placement of the ligature. The ligatures will remain around the tooth for up to 21 days. At certain time points before the 21 day limit, ligatures will be removed by cutting the ligature from the tooth from mice under general anaesthesia. After ligature removal, mice will be maintained for a maximum of 6 weeks. Mice may be subjected to further treatments intravenously, intraperitoneally, subcutaneously, intradermally) or intra-orally, oral gavage, and oral swab for a maximum of 14 days but no more than 42 days. Mice may be subjected to imaging under anaesthesia at various time-points.

Equally, mice that are subjected to treatments to induce tumours would receive one of the treatments, not more. In the case of the grafting experiments, grafted mice will not receive additional substance to modify cell behaviour, and those that do will, in almost every case, receive the substance by a single route. Cell behaviour may be monitored via live in vivo imaging.

The majority of experiments will last between one week to three weeks at most. Most experimental mice will be used before 16 weeks postnatal (2 weeks after the end of the 2nd hair follicle cycle). For breeding pairs, they will be replaced at a maximum of 8 months of age for optimal breeding performance. If available, old breeding pairs may be used for trial runs to test out experimental kinetics, such as concentrations and injection intervals of inducing agents, homeostasis challenge procedures. All mice will be culled by schedule 1 procedures. They may be perfused with PBS immediately after euthanasia, depending on the tissues harvested. Tissues will be processed immediately for flow cytometry or histology.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect intraperitoneal injection of inducing agents cause short-term distress to the animals. Prolonged Treg depletion by repeated diphtheria toxin-injections in Foxp3-DTR mice can lead to multiorgan autoimmunity after 21 days post-treatment. This can result in severe weight loss, and with a tissue pathology characterised by a marked lymphocytic and mononuclear infiltration in multiple organs, such as lung, liver and epidermis of skin. Hence, most of the Treg depletion experiments with homozygous Foxp3-DTR mice will be performed within a duration as short as possible, and no longer than 21 days. Of note, in some occasion, tissue fibrosis may be a necessary output for us to determine Treg functions. Under those situations, a longer period (>14 days of treatment) will be required to develop any pathology or overt signs of mouse distress. We will use female heterozygous Foxp3DTR mice, which will only have half of their Tregs depleted. From

previous experience, no major phenotypes or abnormal behaviour are observed in these heterozygous mice.
However, we will closely monitor these mice (more so than other homozygous Foxp3DTR mice but treated for only a short time frame), to ensure no adverse effects are observed. Any signs of distress, such as a 15% weight loss, hunched posture, inactivity, respiratory problems, will be met with an immediate humane end to experiments.

In skin, we may also challenge the tissue by inflicting different types of skin wounding by tape striping and full thickness wounding using biopsy punches. This may cause short term pain immediately during the procedure, and some discomfort to the animals during healing process.

Mice will be monitored closely for adverse responses (reduced consumption of food or water; sensitivity to handling, piloerection or persistent hunched posture). Any animals exhibiting these phenotypes for more than 2 days will be killed (Schedule 1), or any mice showing clinical signs combined with weight loss of 15% will be humanely killed. Mice with a weight loss of >20% without any other clinical signs will be humanely killed. In the unlikely event (less than 1%, in our experience) of post-operative complications, namely failure of the wound to stop bleeding within 2h or infection; and respiratory problems (abnormal breathing measured by change in rate, depth or pattern for more than 24 hours) in the lung bleomycin model; reduced chow consumption and weight loss in oral injury models etc will be killed unless, in the opinion of the NVS and/or HOI , such complications can be remedied promptly and successfully using no more than minor interventions.

Another adverse effect is in our cancer models in skin and oral cavity. In skin, papillomas or other types of skin tumour induced by DMBA/TPA should be no larger than a combined diameter of 1 cm. Whereas skin tumours can readily be measured, oral tumours may not be visible (for example, if they form in the oesophagus) or may physically distort the mouth. A tumour that cannot be measured will be judged to have reached a humane endpoint if the mouse loses weight, or other clinical signs previously mentioned. If tumour growth results in distortion of the cheek or misalignment of the jaw to such an extent that the mouse cannot close its mouth properly or its teeth overgrowth these will be humane end points.

To our knowledge, the tumour induction protocol should not affect the immediate health of the mice and can be maintained normally. However, if that tumour burden is reached or tumour is grown on sites leading to the discomfort of mice (such as around the eyes, anus, paws), mice will be immediately humanely killed. Besides tumours, no ulceration or other clinical signs are expected. However, if any animal shows distress or pain reaching a moderate severity limit (sensitivity to handling, piloerection or persistent hunched posture) or weight loss of 15%, they will be humanely killed. If a mouse shows signs of infection, antibiotics will be administered, and in the event that the infection is not controlled by antibiotics within 3 days, the animal will be culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We estimated all the breeding of genetic altered animals are of mild severity. About 90% of mice will be injected with gene inducing agents or dissolving agent (such as corn oil, PBS,

saline solution or acetone). About 43% will be used to established cancer models, using cancerogenic agents due to the high variability. About 46.5% of mice (52% of all induced genetic modified mice) will be challenged experimentally with moderate severity, such as tape stripping, hair depletion, gingiva injury, bleomycin transfer etc. Remaining 10% of mice will be wildtype mice without any inducing agents. Of which, 5% of total mice (50% of the untreated wildtype mice) will be control, and the other 5% will be challenged to set the baseline of the immune cell dynamics under genetically unaltered conditions.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Immune-stem cell interactions within tissues is a highly complex line of investigation. However, it is only recently that emerging body of literature has suggested the clinical significance of these interactions.

However, the mechanistic role of immune cell interactions with stem cells during tissue pathology is unknown. Moving directly into in vitro models (directly co-culturing human immune cells with stem cells or organoids) without taking into account potentially unknown factors within the in vivo system would be unwise and an inefficient use of our biological research to address our experimental hypotheses.
Importantly, a detailed survey in the phenotypic and functional characteristics in vivo are required before the establishment of reliable in vitro models. Additionally, while in vitro models can be useful to study molecular signalling of cells, they may not be ideal to study homeostasis, or tissue repair, as multiple cell populations are likely to be involved. Mice are one of the most well-established mammalian models, making them highly biologically relevant to study human diseases with a tissue origin.

Which non-animal alternatives did you consider for use in this project?

Co-cultivation of immune cells with stem cells or organoids, for both mouse and human. Human tissue approaches whereby immune cell stem cell interactions can be studied in healthy and diseased tissues from skin inflammatory disorders, lung fibrosis (idiopathic pulmonary fibrosis) and periodontitis using RNA-sequencing and immunohistology.

Why were they not suitable?

The goal of the project is to understand how barrier tissue resident immune cells, including regulatory T cells, influence epithelial stem cell dynamic changes during normal homeostatic regeneration as well as during the initiation, peak, and resolution post barrier challenge. We are able to study some cell state transitions in co-culture experiments between immune cells and stem cells and validate our findings by examining healthy and diseased barrier tissues in human and mouse. However, in vitro assays and even organoids cannot adequately mimic the coordinated activities of multiple cell types, including cells of the blood vessels, immune system and connective tissue. In addition,

there is an enormously complex cellular interaction and dynamic movement of cells which cannot be accurately modelled in even the most complex in vitro models. Furthermore, culture or organoid models cannot currently recapitulate all aspects of stem cell-mediated regeneration or the multiple stem cell compartments of the skin, lung or oral cavity to enable us to follow the fate of cells differentiating into multiple lineages.
For these reasons we need to perform research on and examine skin, lungs and oral cavity in living animals. We have chosen mice because they are the lowest form of mammal that are adapted to living under laboratory conditions and can be genetically modified for functional studies. In addition, most the immunological studies conducted in the last 12 years has been performed in the strain we have chosen (C57L/B6). Thus, the baseline cellular behaviour of immune cells and stem cells is well known to investigators in the field. This allows us for example, to compare and contrast changes among skin, lung or oral cavity with intestine, a barrier organ where tissue resident immune cells have been better characterised.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 guiding principle in experimental design is to use the minimum number of mice to provide the maximum amount of information. To account for biological variation between mice of the same genetic variation, for most experiments triplicate mice will be used per condition (including experiments on grafting tumour cells), to detect significant differences between conditions. Once a significant difference is identified, the same experiments will be repeated twice again, making up to 3 times with 9 mice per conditions per experiment in total. This is to increase the statistic power of publishable data and minimise technical error. Of note, we will not repeat experiments that we decide not to pursue after the initial experiment. For single cell RNAseq analyses, 2 biological replicates will be used, since the large amount of data usually meant that we only need to ensure there is sufficient duplication in pattern of clustering before formal validation by protein and qPCR as necessary. In validation studies, we will use the amount we use for immunological studies, which is 5-6 animals in 2-3 experiments. However, skin chemical carcinogenesis (protocol 3) is an exception. Typically, we will use a cohorts of 25 age and sex matched mice per condition per experiment. This is because the hair growth cycle affects tumour susceptibility, as does fighting and hormone status, and the number of tumours per mouse can vary considerably.

Another factor depends on number of control conditions we include per experiment. For example, to study the functions of Notch ligand Jagged1 expressing Tregs during homeostasis, we conditionally knockout of Jag1+Tregs using a tamoxifen-inducible Treg specific promoter (Foxp3creERT2 Jag1f/fl). Tamoxifen will be prepared by conventional method: dissolving in corn oil and sonicated before intraperitoneal injection in mice. 3 types of controls will be employed: (i) untreated transgene-negative littermates (wildtypes), to set the baseline, (ii) tamoxifen-treated wildtype, to test the tamoxifen toxicity, and (iii) corn oil treated Foxp3creERT2 Jag1f/fl, to ensure no predisposed alteration in Jag1+Tregs populations prior to tamoxifen induction. When these conditions are established, all these

3 controls should provide us with the same phenotype, i.e. a similar proportion of Jag1+Tregs. However, these duplications are necessary repeats to ensure tamoxifen induction is working correctly in independent experiments. Thus, it will require 9x4 = 36 mice per Foxp3creERT2 Jag1fl/fl experiment.

An important consideration will be the predicted genotypes of the offspring arising from each pregnant female. In some case, heterozygous breeding pairs are kept generating littermates without genotypic alteration as controls for experiments. Hence, more pregnant mice are required as only 25% or 50% will have the desired genotype.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To avoid unnecessary generation or import of genetically altered animals we will search cryobanks and databases. Examples of resources available include: NC3R's mouse database: https://www.nc3rs.org.uk/minimising-use-ga-mice. Animal Welfare Management Discussion Group (AWMDG), Mouse locator: Locator@cancer.org.uk, PubMed: http://www.ncbi.nlm.nih.gov/, Web of Knowledge: http://wos.mimas.ac.uk, Jackson laboratory: http://www.jax.org/, http://jaxmice.jax.org/index.html, Cre transgenic database, see PMID: 19266338 "Creation and Use of a Cre Recombinase Transgenic Database.

We will have a digital system in place for genetically modified colony management.

Furthermore, our hypotheses are generated via multiple non-regulated procedures: (i) analysis of human tissue sections (healthy and diseased), (ii) analysis of published mouse mutants, (iii) analysis of gene expression and high dimension genomic data, from our own lab and other published data, and (iv) computational models of tissue homeostasis, wound healing and cancer. At least two methods will be employed before we move into in vivo models.

We have consulted in-house statisticians in order to perform statistical power calculations to determine the minimum number of animals needed to complete these studies and give statistically relevant results. Prior to performing an experiment, we perform statistical analysis to ensure that we use the minimum number of mice per group that will be informative. We aim to achieve a minimum power of 0.8, assuming statistical significance of $P < 0.05$. Initially, we will test for normality and distribution. For normally distributed data we will employ a parametric test (e.g. ANOVA), otherwise a non-parametric test such as Kruskal-Wallis one-way analysis of variance or Mann Whitney analysis will be used. We will also consult our in-house statisticians when needed. We will comply to the ARRIVE guidelines including blinding and randomisation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To prevent unnecessary breeding, we will keep stocks of frozen mouse sperm and embryos of each strain, and cull those not actively used for experiments. If available, most pilot experiments will be conducted on ex-breeders or mice with undesired genotypes. We will harvest all three barrier tissues (skin, lung and oral cavity) of the same wildtype or genetic altered mice, as these experiments can run in parallel. Furthermore, all genetic alterations will be bred into C57BL/6J genetic background, to minimise genetic background variability. All experiments are using mice with age and sex matched, and littermates lacking one or more of the transgenes as controls in experiments.

During histological analysis of tissues, we are developing a tissue bank of all barrier tissues from mice of different ages, and those challenged. Each harvested tissue is split to three and preserved in paraffin, OCT and RNAlater solution (which stabilises and protect cellular RNA for molecular analysis).

Furthermore, we plan to use these tissues to generate a tissue microarray, by cutting 60 µm dimeter serial sections which will allow us to perform multiple histological analysis of many (more than 6) markers without sacrificing additional mice. Importantly, this will allow us to have a more credible identification of the cell populations spatially but also a better comparison between different conditions. Critically, this will optimise the number of mice used, and be used across multiple projects to set the baseline of immune cells and other tissue resident cell populations with respect to exact tissue localisation.

Whenever possible, we will use grafting human tumour cell instead of chemical carcinogenesis. This can reduce the likelihood of rejection and animal suffering, but also a more controlled tumour formation. For example, grafting tumour cells in skin yield nearly a 100% success rate, whereas the results from chemical carcinogenesis may vary. Thus, the number of mice per condition for grafting is 3 mice, while chemical carcinogenesis can be a maximum of 25. However, under some situations (such as studying the dynamic of tissue resident immune cells during cancer manifestation), chemical carcinogenesis is required to model the transition from health barrier tissue to cancer bearing condition. For that, we use the same parental mice for the first and repeat experiment. This will minimise genetic variations and reduce the amount of mouse breeding required per experiment.

Additionally, we will also share necropsy samples (such as spleens, lymph nodes, bone, pancreas, intestine) with other research groups, so that they can obtain data without having to breed their own mice. It is anticipated that advances in non-invasive imaging technology will potentially reduce the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the lowest form of mammal that can be used to study normal and diseased barrier tissues. They are extensively studied and have a huge range of developed molecular tools to manipulate their genome precisely. They are the only mammal in which transgene technology works reliably. In fact, with the advancement of mice breeding technology, laboratory mice can be inbred to yield genetical identical strains, minimising background genetic variations between individuals and maximising the credibility of experimental results.
Many parameters and protocols are already defined, reducing the amount of optimisation required. We will consciously refine our experimental set up to reduce the suffering of the animal and improve their welfare. For wounding experiments, we will use previously

published score sheets to measure the healing process. After bleomycin instillation (lung fibrosis model), which will be instilled non-surgically via intra-tracheal administration, as opposed to surgical incision to the trachea, animals will be checked regularly for clinical signs and pain using a clinical score, as a reference. Oral squamous cell carcinoma (OSCC) development (4NQO in drinking water) will be visualised for progression for hyperplasia followed by dysplasias (demonstrated by formation of raised, white lesions) and then full development for OSC. This model is slow progressing, however, regular monitoring of the oral cavity with reference to the aforementioned study will determine the end-point and reduce further suffering to the animal. As for the ligature-induced periodontitis model, this is a relatively non-invasive procedure, we can reduce suffering by regular monitoring the animal's health. Previous publications indicate alveolar bone damage can occur in as little as 6 days, plateauing between 11-16 days. With this in mind, we aim to remove ligatures by 21 days and terminating the experiment by 6 weeks.

When a new drug is being tested, initial experiments will be carried out with a small number of mice. The conditions tested will be predicated from the literature, to ensure the drugs does not result in generalised adverse effects. The data for the first trial experiments will inform us the design of subsequent experiments, which includes larger number of mice. Blood sampling will also be carried out in some experiments to monitor the circulating immune populations, drugs and/or other metabolites, in response to the challenge or treatments.

Dosing volumes used will be the minimum practicable depending on the solubility of the agent and accuracy of administration and will be adjusted according to individual animal body weights at each dosing. The frequency of dosing will be balanced against the duration of the treatment schedule such that the total number of doses administered is not excessive. Maximum dosing volumes and frequencies for conscious treatments or under anaesthesia are in accordance with published guidelines. Maximum overall number of injections to be given by any route: A maximum of 14 injections in one week, 28 injections in two weeks and 42 injections in three weeks or more.
Maximum volumes in any one injection will be according to the LASA Good Practice Guidelines (http://www.verutech.com/pdf/lasa_administration.pdf)

In the case of administration of agents, the following Table of maximum dosing volumes and frequencies will be used. These maximum volumes and numbers of injects by different routes are normally well tolerated, assuming substance injected is non-toxic.

Maximum frequencies of injections

Maximum volumes in any one injection

Route	1 per day	2 per day	1 per week	2 per week	3 per week
Route	Mouse		Rat		
subcutaneous	6 weeks	3 weeks	no limit	no limit	12 weeks
subcutaneous	20ml/kg body weight		5ml/kg body weight		
intradermal	6 weeks	3 weeks	no limit	no limit	12 weeks
intradermal	50µl per injection		100µl per injection		
intratumoral	6 weeks	3 weeks	no limit	no limit	12 weeks
intravenous (low volume)	10ml/kg body weight		5ml/kg body weight		
intravenous*	6 days	2 days	12 weeks	6 weeks	2 weeks
intravenous (high volume injection)	100ml/kg body weight		100ml/kg body weight		
intraperitoneal	6 weeks	3 weeks	no limit	no limit	12 weeks
intraperitoneal	20ml/kg body weight		10ml/kg body weight		
oral gavage	6 weeks	3 weeks	no limit	no limit	12 weeks
intratumoral	50µl per injection		100µl per injection		
intranasal	6 weeks	3 weeks	no limit	no limit	12 weeks
intramuscular	50µl per injection site		50µl per injection site		
intramuscular	Not more than once a week into both hind legs for a maximum of 3 weeks.				
intranasal	50µl per injection		50µl per injection		
oral gavage	20ml/kg body weight		20ml/kg body weight		

(* including administration of substances and collection of blood samples)

	s/c	i/p	i/m	i/v bolus	i/v infusion	Oral gavage
Maximum number	24	24	6*	14	14	20
Maximum daily volume ml/kg	20	20	-	5	20	25
Max for a 25g mouse	0.5ml	0.5ml	0.05ml	0.12ml	0.5ml	0.6ml
Maximum number per day < 7days	3	2	2	2	2	2
Maximum number per day > 7days	2	1	1	1	1	1

Why can't you use animals that are less sentient?

The goal of the project is to understand how barrier tissue resident regulatory T cells and other tissue- resident immune cells influence epithelial stem cell dynamic changes during normal homeostatic regeneration, post injury and diseased barrier tissues. We decide to use juvenile and adult mice due to the biological relevance.

For skin: For skin: In mice, hair follicles (HFs) actively cycle, self-renew, and generate new hair shafts through a repeated cycle of shedding, quiescent and growth phases. For C57BL/6 mice, their HFs begin to form right after birth, but only enter their first quiescent phase at 3 weeks of age, and growth phase at 4 weeks, suggested by previous literature. Importantly, it has been commonly acknowledged that the first two HFs cycles (1st cycle: 3 to 6 weeks postnatal (juvenile), 2nd cycle: 7 to 14 weeks postnatal (adult)) are highly synchronised in C57BL/6 mice, making the first two cycles perfect model to study hair growth. Furthermore, previously it has been shown skin Tregs mediate this HFs transition from quiescent to growth phase. Hence, it is critical we have a model capturing the transition. However, since after the 2nd HF cycle, the hair growth are no longer synchronised on the dorsal skin. In other words, certain locations may enter quiescent phase but not other locations. It becomes difficult to study the dynamics of skin resident

immune cells with hair regeneration. Thus, we will limit the age of our samples in neonate, juvenile and adult only.

For lung: We aim to study the lung resident immune cell and stem cell dynamics of mice at adult and P5-P30 postnatal stages, after the lung has been fully developed and become functional. The identification of a particular condition/ environment produced by the TRICs during alveologenesis at a steady state, may inform us a potential strategy to promote alveolar regeneration in a fibrotic setting in adult mice. To the best of our knowledge, we are not aware of any studies that administer bleomycin to neonatal mice, therefore we will use adult mice with a fully developed respiratory system which are commonly used.

For oral: In vivo mouse models of oral disease used in this study have been extensively used in research given their ability to partially reflect the diseased state observed in humans. Therefore, we will be inflicting local injury using the aforementioned models on live mice as opposed to terminally anesthetised mice, to study the immune cell and epithelial stem cell dynamics upon steady state and diseased state. We also aim to study the dynamics of oral tissue resident immune cells and oral epithelial stem cells during different ages of mice such as neonatal, juvenile and adult mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most the homeostasis challenging procedures and cancer inducing models will be done with local or general anaesthesia (AB), either through inhalation or as a short acting injection. Pre- and post- operative analgesia will be provided; agents will be administered as agreed in advance with the a veterinary surgeon (NVS). Majority of anaesthesia will be short acting (<2hours). However, for in vivo live imaging, prolonged anaesthesia may be necessary. In that case, supplementary heating and fluids will be provided as necessary. Mouse body temperature will be monitored. When mice are being imaged in a chamber associated with a microscope the level of hydration of the chamber that encloses the microscope stage will be monitored. If a mouse shows signs of distress (e.g. fall 2 degree C in body temperature, shallow breathing) the imaging session will be terminated and the mouse will be allowed to recover on a heated pad and provided with food and water.

Unless otherwise specified, all animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Uncommonly animals that fail to do so or exhibit signs of pain, distress or of significant ill health should be humanely killed by schedule 1, unless in the opinion of NVS, such complications can be remedied promptly and successfully using no more than minor interventions. Any animal not fully recovered from the surgical procedure within 24 hrs (eating, drinking and return to normal behaviour) should be humanely killed.

A programme of enhanced monitoring and care is instituted until the animal fully recovers. For example, after cell injection, mice will receive analgesia. They will be monitored closely (hourly for first two hours then daily)for adverse responses (reduced consumption of food or water; sensitivity to handling, piloerection or persistent hunched posture). Any animals exhibiting these phenotypes for more than 2 days will be killed (Schedule 1), or any mice showing clinical signs combined with weight loss of 15% will be humanely killed. Weight loss in the absence of any other clinical signs will result in humane killing, or in the case of individual animals of particular scientific interest, advice will be sought promptly from the local Home Office Inspector. If mice show signs of infection they will be treated with antibiotics and will be killed within 3 days if they show no signs of improvement.

Furthermore, the adverse effects of bone marrow transplantation will be minimised by treating mice with acidified water prior to irradiation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Dosing volumes used will be the minimum practicable depending on the solubility of the agent and accuracy of administration and will be adjusted according to individual animal body weights at each dosing. The frequency of dosing will be balanced against the duration of the treatment schedule such that the total number of doses administered is not excessive. Maximum dosing volumes and frequencies for conscious treatments or under anaesthesia are in accordance with published guideines. Maximum overall number of injections to be given by any route: A maximum of 14 injections in one week, 28 injections in two weeks and 42 injections in three weeks or more.
Maximum volumes in any one injection will be according to the LASA Good Practice Guidelines (http://www.verutech.com/pdf/lasa_administration.pdf)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

One important part of our research is to create immune cell-organoid systems for all our three targeted barrier tissues, namely skin, lung and oral cavity. For that we will be keeping informed with the most recent literatures of in vitro methods to co-cultivate immune cells with organoids. Upon the success in creating and validating a system which allows us to recapitulate what we observed in vivo, majority of the work (especially the drug development parts during the later period of our research) will be performed with these models. This will greatly reduce the number of mice we used, and ideally replace them completely at one point.

Additionally, we will keep in touch with our animal units, to be updated with any new policy or regulation changes in the NC3Rs. We will consciously refine our experimental set up to reduce mouse suffering.

176. The Autonomic Nervous System in cardiovascular regulation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

kidney, renal afferent nerves, paraventricular nucleus of the hypothalamus, heart, renal

efferent (sympathetic) nerves

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?

Identify the neural circuits by which kidney function is monitored and controlled.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 evidence that abnormal regulation of the nerves that control the heart and kidney leads to high blood pressure and heart failure. The focus to find treatments for these cardiovascular diseases has moved to the kidney because of clinical trials investigating the efficacy of the therapeutic approach of removing the nerves that control blood flow to the kidney. This radical treatment has the potential to reduce blood pressure and improve cardiac function, which the clinical trials do indicate. However, the mechanism behind the improvement in cardiovascular function is not fully understood because removal of the renal nerve interrupts both signals to and from the brain so it is impossible to know which contributes to the effect. Animal experiments suggest interruption of the signals going into the brain may be important but because there is a lack of knowledge regarding how under normal circumstances this information is communicated within the brain it is hard to know what effect the removal of the circuit has. If renal nerve removal is to become a recognised treatment for cardiovascular disease then it is essential to identify the neural pathways contributing to the effect.

What outputs do you think you will see at the end of this project?

We will benefit from a more thorough knowledge of the integrative processes controlling cardiovascular regulation in health and disease. Also, the basic research will give us insight on how a potential new therapy may be enacting its effect. The work will be presented at the relevant scientific conferences as well as published in peer review journals.

Who or what will benefit from these outputs, and how?

If renal denervation is to become a recognised treatment, then the basic research outcomes have the potential to refine a new therapy to treat cardiovascular disease. For patients and society, there is the benefit of better treatment strategies to improve mortality rates but also the potential to tailor interventions depending on the stage of the disease and or improve preventative interventions.

How will you look to maximise the outputs of this work?

We will look for relevant expert collaborations and share methodological innovations that should encompass unsuccessful approaches

Species and numbers of animals expected to be used

- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are looking at systems interaction and behaviours and how these are altered in complex diseases. To understand these coordinated mechanisms requires an animal model.

Typically, what will be done to an animal used in your project?

Protocol 1: Anaesthetised for recovery surgery. Undergo surgery to expose an autonomic structure which will be injected with a neuronal tracer. The animal will recover from this surgery. A period of up to 3 months to allow the tracer to travel to its end target will follow, when the animal will be reanaesthetised and a further autonomic structure injected with a neuronal tracer. Again, recovery for up to 4 weeks. Terminal anaesthesia when the animal will be perfuse-fixed or kidney exposed for stimulation (up to 3 hrs) of this structure and then following this perfuse-fixed. Or terminal anaesthesia removal of "live" tissue for molecular biological manipulation of pefused-fixed material for immunohistochemical processing.

Typically 90% of animals will undergo 2 recovery anaesthetic interventions with remaining 10% receiving 1 recovery anaesthetic intervention before proceding to terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection into autonomic structures may result in temporary discomfort and modified behaviours, such as weight loss, piloerection, dehydration, hunched appearance, solitary or subdued behaviour (<5%).
Should last no more than 48hrs

Injections into the spinal cord may cause temporary hind limb paralysis (one or both) on injected side (<5%). Should last no more than 48hrs

Expected severity categories and the 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?

We are looking at systems interaction and behaviours and how these are altered in complex diseases. To understand these coordinated mechanisms requires an animal model. Previous research has shown that rodents can give insights into human diseases and can lead to novel therapeutic development.

Which non-animal alternatives did you consider for use in this project?

Computer modelling, ex vivo tissue and tissue culture methodologies.

Why were they not suitable?

Current computer modelling tools lack the level of complexity to give meaningful data. Ex vivo tissue sampling can be used for experimentation but tissue still has to be derived from an animal. Tissue culture can provide data on how a neurotransmitter might function however, how that function is integrated into a complex behaviour cannot be determined.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates based on my wealth of experience coupled with mine and others published literature gained over 25+ years. Estimates are informed by an in-depth knowledge of the procedures involved and experience of previous licences in this area of research.

What steps did you take during the experimental design phase to reduce the number of

animals being used in this project?

Advances in knowledge and understanding, gained through my own and others' work in this area, position this licence to require a reduced number of animals and species than the one it is replacing. From experience, power calculations do not lend themselves to neuroanatomical investigations as there are numerous steps where error can be introduced. An in depth understanding of the procedures gained from experience of carrying out such work contributes to the reduction in animals required. For example, surgical procedures are carried out in groups of 4-6 to reduce biological variation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For neuroanatomical labelling and subsequent tissue processing, the number of sites where error can be introduced is numerous. For example, precise injection of tracer into the CNS is required.

Biological variation in animals can mean the injection does not quite hit its target. For tissue immunohistochemistry requiring antibodies there can be considerable variation in antibody efficacy from commercial suppliers, coupled with the incubation steps required to utilise the antibody introduce further possible places of error. Animals are used in groups of 4-6 thereby reducing biological variation for injection site. Antibodies from known batches and sources are utilised and tissue from each animal can be processed independently to minimise failure.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 looking at system interaction and behaviours and how these are altered in complex diseases. To understand these coordinated mechanisms requires an animal model. Rats are chosen as a widely used cardiovascular model to investigate cardiovascular regulation in humans. A lot is understood about the control of the cardiovascular system in the rat, meaning any new information can be understood without having to repeat experiments in new species. All surgical procedures are carried out by trained individuals and are carried out in sterile conditions and using techniques to minimise the time in surgery, reducing the risk of infection and prophylactic use of analgesia and antibiotics where applicable.

Why can't you use animals that are less sentient?

The dyes used to trace nerve pathways work most efficiently and effectively in freely moving living systems. More animals would need to be used if the tracing studies were carried out in terminally anaesthetised animals as this methodology greatly slows the transport of the dye hindering the chances of success. The complete connectivity of the nervous system is not complete in immature life stages therefore older animals are

required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All individuals will be trained to carry out the surgical procedures. Observation of behaviours during recovery from surgery will provide information about the welfare of the animals and can inform prophylactic use of analgesia and antibiotics. Keeping animals in their social groups

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

One guidance source will be ARRIVE which has just been updated. NC3R sources

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Monitor the NC3R website and engage with their resources. Work with the animal technicians and take advantage of their forum networks which share information. Any relevant advances will be tried alongside existing practices and compared to evolve the best approach to achieve the aims of the work

177. New treatments for right ventricle in pulmonary arterial hypertension

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
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Right heart, Sex difference, Pulmonary arterial hypertension, Therapeutic targets, Mitochondrial function

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 determine the difference between males and females in the mitochondrial function (the mitochondria are the energy engines of the cell) of the right side of the heart with the aim of identifying new ways to treat pulmonary arterial 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?

Pulmonary arterial hypertension (PAH) is a disease that causes high blood pressure in the arteries that go from the right side of the heart to the lungs. PAH is a rapidly progressive disease resulting in right heart failure and death. The 5-year survival is 37%, and over

15,000 deaths/year are ascribed to PAH. Though right heart function mainly determines the clinical outcome of patients with PAH, no effective therapy directly treats the right heart in PAH. It is known that there is sex difference in PAH: 3-4 times more women than men develop this disease, but women have better right heart function and thus survival rates than men. In addition, right heart failure is related to mitochondrial dysfunction.

Mitochondria, the powerhouse of the cell, is an important cellular component that not only produces about 90% of energy that cells need to survive but also regulates other cellular functions. The effect of sex hormones on the mitochondrial function in the right heart in PAH is not well studied. Currently the availability of right heart tissues from human PAH patients is limited. Instead, this research program will make extensive use of well recognized and often used method of studying PAH in rats. This project will study the sex difference in the mitochondrial function in the right heart in the animal model and identify novel potential therapeutic targets for the right heart in PAH.

What outputs do you think you will see at the end of this project?

Several outputs are: (1) Sex-dependent mitochondrial function in the right heart in the animal model of PAH will be well determined; (2) Novel potential therapeutic targets (possible new treatments) for the right heart in PAH will be proposed for further clinical trials; (3) Several peer-reviewed articles will be published in scientific journals.

Who or what will benefit from these outputs, and how?

In general outputs from this project will inform clinicians and other scientists of novel potential therapeutic targets on the mitochondria (the energy engines of the cell) in the right heart in PAH of both males and females.

In the short term our work will first provide data on the expression of mitochondrial and mitochondria- related genes and proteins in the right heart of both males and females in a gold-standard animal model of PAH, this information will be shared via published papers and will give scientists in the field a clearer understanding of the role of mitochondria. In addition, the cellular mechanism of potential targets will be revealed and understood, clinician-researchers will use this knowledge to guide their research via focusing on these potential targets in both in vitro cellular studies and pre-clinical animal studies. If further funding is obtained we will extend the project to 5 years and the benefits will continue to medium goal.

In the medium term the studies will build on information gained to encourage the development of clinical trials to establish the effectiveness of any possible treatments.

In the longer term, patients will benefit through novel therapies becoming available if full clinical trials are successful.

How will you look to maximise the outputs of this work?

We will present our new data or findings in seminars and national and international conferences, and publish the new knowledge in scientific journals. We will also collaborate with clinicians and scientists to develop clinical trials if potential therapeutic targets are identified.

Species and numbers of animals expected to be used

- Rats: 120

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adults rats will be used to mimic the effects of PAH so that it can be studied. The use of rats in the way is well-accepted for a study of this kind.

Typically, what will be done to an animal used in your project?

The procedure will involve a single injection of a drug. This is followed by up to 3 weeks of hypoxia (a chamber which reduces the amount of oxygen, similar conditions to being on top of a high mountain) and then up to 8 weeks of normal oxygen intake to develop PAH. The heart will be monitored using an echocardiography. Finally, under anaesthesia that the animal does not recover from a catheter (tube) will be inserted to measure blood pressure in the animal's heart (this is a non-recovery procedure).

What are the expected impacts and/or adverse effects for the animals during your project?

Animals with the combination of injection of drug and hypoxia gain less weight compared to an age- matched control animal and develop PAH. Animals are used usually before they show any significant clinical signs, apart from the lack of weight gain compared to controls. If significant signs of ill-health (due to PAH) are found, the animal will be immediately used with a terminal procedure (echocardiography and catheterization) if possible, or humanely killed by an approved Schedule 1 method.

Echocardiography: is a non-invasive procedure. Minimal temporary discomfort may occur during the induction of anaesthesia. Anaesthesia is used just to keep the animal still for accurate recording so the depth of anaesthesia does not need to be very deep, it will be continuously monitored throughout to ensure the animal remains unconscious with respiration rate and rhythm monitored.

Hypoxic exposure (reduction of oxygen): The animals within the hypoxic chamber will be monitored daily. In the animals that will be allowed to recover from pulmonary hypertension, there are no adverse effects anticipated. Animals lose body weight within the first few days of hypoxic exposure and demonstrate reduced activity. However, acclimatisation then occurs with animals returning to normal activity and eating behaviour, and they begin to gain weight. They generally remain below an age matched control, however this weight loss & any associated with drug administration will not be allowed to fall below 20% of an age matched control animal.

At the end of the study when the animal is under deep anaesthesia catheterisation takes place: this is a non-recovery procedure. Minimal temporary discomfort may occur during the induction of anaesthesia. From previous studies, the anaesthetic death rate is expected to be less than 1%. Depth of anaesthesia will be continuously monitored throughout to ensure the animal remains unconscious and insentient.

This will be assessed by pedal withdrawal reflex (which indicates whether the animal is feeling pain) and monitoring of respiration rate and rhythm. If haemorrhage from blood vessels or heart occurs, attempts to control it will be made by pressure or cauterisation. If this cannot be controlled the animal will be killed (schedule 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 severity will be moderate for animals injected with drug followed by oxygen reduction, and it will be mild for control animals injected with vehicle solution followed by exposure to normal levels of oxygen.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 PAH patients the changes in the lungs cause the right side of the heart to work harder and eventually the patients die of right heart failure. Patients are already very ill when they are diagnosed and so experiments cannot be carried out on them. We can only study the function of the heart in a living animal. Therefore, animals are essential if we are to find new drugs to save the lives of these patients.

Which non-animal alternatives did you consider for use in this project?

Where possible information will be gained from examining the right heart tissues or cells from the right heart from PAH human patients and normal humans, however, access to these tissues and cells are limited.
I also thought of working with in vitro mitochondria (the component I am interested in inside the cell), however, it is currently very difficult to maintain and keep mitochondria viable for the period required in a laboratory environment. Also they do not maintain the same phenotype (observable characteristics or traits of an organism) in culture.

Why were they not suitable?

Right heart tissue or cells from the right heart from PAH or normal human patients are not available. The cells from animal models are not commercially available. Even though we may obtain cell lines from normal animals either commercially or from other research groups, the study of only normal cells that is performed outside of a living organism is not that useful. The right heart is complex with several cell types. The study of normal cells produced outside a living organism is not able to replicate the conditions within the heart nor does it provide enough information about the structure and function of the right heart.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 using published data in scientific journals we estimate the minimum number of animals that we need to give the difference in the data we will measure between normal animals and animals with PAH. Only this data would be considered by clinicians when looking at new therapies to put through a clinical trial on patients. The number is calculated based on the changes we wish to measure. Wherever possible we would never repeat experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

NC3R's EDA and advice from named statistician from Host Institution. The number required to reach difference between groups will be re-assessed with pilot studies in the early stage of the project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In the early stage of the project, pilot studies on 3 or 4 animals per group will be performed to estimate the difference between different groups (animals with PAH and normal animals) and optimize the required number of animals for each group to reach the difference we need. In other words, we may need greater or less number of animals based on the pilot studies than the number that is originally estimated before the start of the project. In addition, the right heart tissue from each animal will be divided to several parts for different measurements. Moreover, since this project will focus on the heart, the lung tissues will also be saved and shared with other researchers who are focusing on pulmonary arteries in the lungs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the animal model which is widely recognized and used in this research, which mimic the aspects of the human disease (PAH) we wish to study.

Why can't you use animals that are less sentient?

The animal model we will use is the gold-standard model of the human disease (i.e., PAH) we wish to study. The model with animals at a more immature life stage, or other species that are less sentient are not relevant to the proposed study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use standard, refined protocols that utilize the minimum dose of induction drugs resulting in PAH without causing significant clinical signs. All animals are studied by fully trained researchers and regularly checked for any signs of ill health. If the animals (especially PAH animals) develop signs of ill- health that can not be easily reversed and it is before the time for scheduled experiments, the animals will be either used in terminal experiments (echocardiography, blood pressure measurement, killing of animals, and tissue harvest) or humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All relevant NC3R Guidelines and papers within the field of cardiovascular research

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Check the NC3Rs and Norecopa website regularly. I will also get information from the Named Information Officer (NIO). I will stay up to date with NC3Rs Animal Research: Reporting of in Vivo Experiments (ARRIVE) guideline to check for any update and implement them if relevant to the project. I will attend conferences etc. to keep up to date with advances in the field.

178. Pathophysiology of Cardiovascular and Metabolic Diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cardiovascular Disease, Non-alcoholic steatohepatitis, Heart Failure, Biomarkers, Therapy

Animal types	Life stages

Mice	adult, embryo, neonate, juvenile, pregnant
Rats	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project has several objectives that fall under one or both of two over-arching aims:

Identification of novel biomarkers to determine the risk of cardiovascular disease or non-alcoholic steatohepatitis (NASH) in the setting of obesity/metabolic syndrome or micronutrient dysregulation.

Identification of novel drug targets, and assessment of novel pharmacological agents directed at them, for the prevention of cardiovascular disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Heart and circulatory disease (Cardiovascular Disease; CVD) manifests in various ways, including high blood pressure (hypertension) and narrowing of the arteries (arteriosclerosis), which can lead to heart attack (acute myocardial infarction; AMI), heart failure and stroke. Despite a global reduction in the incidence of CVD, death rates from CVD in the UK remain amongst the highest in Western Europe and is the UK's biggest killer; statistics for 2020 show that 7.4 million people in the UK are living with CVD, and around 27% (170,000 people) of all deaths in the UK result from CVD. Although there are therapies that can help to control blood pressure and the development of arteriosclerosis, there are few (if any) therapies that can reduce the tissue injury that results from AMI or stroke. Consequently, for those living with CVD there is decreased physical fitness and increased morbidity.

Approximately 80% of individuals with CVD have at least one other health condition linked to the development (such as metabolic syndrome or hypertension) or as a consequence (e.g. heart failure) of CVD. Metabolic syndrome (MetS) refers to a cluster of metabolic disorders associated with visceral obesity, insulin resistance, dyslipidaemia, hyperglycaemia, and hypertension, predisposing individuals to increased risk of arteriosclerosis, CVD, Type 2 diabetes (T2D) and non-alcoholic steatohepatitis (NASH). MetS is an even greater global public health problem, affecting 25% of the world’s adult population, with a similar prevalence reported for the UK. As no single treatment to control

MetS exists, novel therapeutic strategies are required to improve population health. Therefore collectively, there is a continued need to find novel ways of preventing (e.g. through dietary intervention), detecting risk of developing (e.g. through detection of biomarkers) and treating (through novel therapies) CVD and its associated risk factors.

What outputs do you think you will see at the end of this project?

This project will provide new information about the cellular events by which dietary risk factors such as obesity, metabolic syndrome and micronutrient deficiency lead to the development of coronary heart disease, heart failure and fatty liver disease (co-morbidities). This information will give an indication of where an intervention (for example a drug or nutritional supplement) targeting these events could prevent the development or halt the progression of these co-morbidities. Furthermore, it will identify novel biomarkers that can be used to either predict the likelihood of developing a co-morbidity or to determine the effectiveness of treatment.

This information will be shared with the wider scientific community through conference presentations and published as scientific articles in peer-reviewed, high quality, open-access journals. Identification of drug targets, and novel compounds acting at them, or of novel biomarkers, may lead to the generation of intellectual property that could be licensed to the pharmaceutical industry for commercial development. In addition, identification of new dietary interventions to promote health and wellbeing could lead to the production of new nutritional supplements or nutraceuticals.

Who or what will benefit from these outputs, and how?

The beneficiaries of this information will be:

Researchers within the field of cardiovascular and metabolic science will be informed of ongoing work through conference presentations(2-3 per year) and eventual publication of the final data arising from different studies within the overall programme (within 6-9 months of completion). All scientific outputs will be in green open-access journals, and will be uploaded into the Institutional Repository in accordance with the University Data sharing policy and any journal- imposed restrictions on access.

The pharmaceutical industry undertaking R&D programmes to develop novel ligands targeting the receptor systems under study; where intellectual property with potential for commercialisation is generated, we will work with the Establishment IP officer to ensure that this is protected (up to 1 year). We will then explore the opportunities for licensing out this IP through discussions with companies working in the same field (~2-3 years from completion of the project).

The clinical community since, although there are limitations to attempting to translate experimental findings into the clinic, the new information could inform hypotheses for subsequent collaborative controlled clinical studies, for example validation of a biomarker to demonstrate the stage of disease progression; we already work with clinical colleagues to acquire patient blood samples from specific patient groups alongside some of our in vivo experimental studies.

The general public, who will be kept abreast of developments through press releases (where appropriate) on relevant research outcomes timed to coincide with the publication of a full article. We also encourage our PhD students to participate in public engagement

activities.

How will you look to maximise the outputs of this work?

To avoid unnecessary replication of data by other groups, where appropriate, raw data arising from the studies will be made available to the wider scientific community in accordance with the Establishment's Data sharing policy and provided as supplementary information for published articles.

We believe that negative data is as important as positive data, and should be published to inform the scientific community. Many peer-reviewed journals now agree with this philosophy, and publish negative studies as long as the underlying science is robust. Therefore, in the event that we fail to generate data to support our original hypothesis we will publish these findings to prevent other scientists from travelling down the wrong route

Collaboration is key to the success of our research programmes, and we already collaborate widely with colleagues in academia both nationally and internationally (e.g. Australia, France, Italy). We also collaborate closely with industries involved in pharmaceutical R&D and those involved in isolation/production of health-promoting bioactives from by-products of raw material processing, such as marine algae and agricultural crops.

Species and numbers of animals expected to be used

- Mice: 1500
- 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.

Rats and mice are well-established models to study the impact of diets aimed at inducing obesity and metabolic syndrome, and also to determine the impact of micronutrient deficiencies, on cardiovascular physiology and tissue structure and function. Since diet-related problems in humans arise as adults, the most appropriate life stage to mimic these events in animals is at the adult stage.

Genetically modified animals that express alterations in genes that code for proteins linked to energy metabolism are important models to use to be able to understand the importance of these proteins in the development of diet-induced cardiac and liver disorders. Although breeding of these animals involves animals at all life stages, but only adult animals will be used for any experimental procedures.

Typically, what will be done to an animal used in your project?

A typical experiment will involve feeding wild type and GM mice a high cholesterol diet for 12 weeks, with weekly measurement of body weight and non-invasive assessment of body composition (lean vs fat content) at the start and end of the diet period. When testing for the effects of a drug intervention, this would be given in the drinking water for the last 2-4

weeks of dietary intervention. 2-3 days before the end of diet intervention, animals will be tested for their glucose status; this involves withdrawal of food (for up to 8 hours) prior to an injection of either glucose or insulin. After the injection blood samples are taken approximately every 10 minutes, over a 1 hour period, via tail prick and blood glucose measured using glucometer strips. At the end of the diet intervention animals are either humanely killed using an authorised method of euthanasia, or terminally anaesthetised for measurement of cardiovascular variables prior to euthanasia.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will not experience adverse effects that are more than mild and transient.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

With the exception of surgical pump implantation, all of the procedures are of mild severity, that is they are of low impact, non-painful, have no lasting effect and exhibit a very rapid return to normal. Around 5% of animals may undergo pump implantation, which is of moderate severity as they undergo surgery under general anaesthesia. However measures such as pre- and post-operative pain relief reduce the impact of surgery and the animals return to normal within one or two 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?

Cardiovascular and liver disease arises from metabolic syndrome or dietary deficiency as a result of complex interactions between tissues and organs within the body. While we can study some of the direct influences of dietary modification in isolated cells, tissues or organs, to gain a full understanding of how changes in one tissue (e.g. fat tissue) influence the normal functioning of another organ (e.g. the heart or liver) means that this can only be assessed in a whole animal.

Which non-animal alternatives did you consider for use in this project?

We perform a broad range of experiments in cultured cells and tissues to understand the key changes, for example alterations in anti-oxidant status or release of biochemical markers, that we would expect to see in response to alterations in the conditions aimed at mimicking dietary insufficiency or simulating metabolic syndrome. Where suitable genetic strains of the roundworm, C. elegans, exist we perform experiments to assess certain events, such as fat accumulation in response to metabolic stimuli. Only once we have gathered sufficient information to justify the need for in vivo studies will we use animals.

Why were they not suitable?

Cell-base or non-mammalian in vivo models (i.e. C. elegans) can provide a wealth of information. However, because they do not allow the study of how different organs/tissues in the body respond collectively to changes in diet and metabolism (cells) or do not have the same physiology as mammals (including humans) they cannot provide the full picture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 many years of experience of undertaking dietary intervention studies and so we can use our previous data to determine how many animals will be required to obtain scientifically sound data upon which we can form solid conclusions; this will take account of the inherent biological variability for the desired end points that will be measured. In addition we can gain further insight with regards to experimental group sizes from the wider scientific literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In addition to using previous data to inform our decisions around the number of animals to use in any particular study, we also make use of online tools, such as the NC3R's Experimental Design Assistant, and follow the ARRIVE guidelines on designing and reporting experiments using animals. We also seek advice from the Establishment statistician when required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For studies using GM animals we only breed the numbers required for a particular study; where possible we utilise both male and female animals unless there is a clear scientific reason to only perform studies in one sex. When we perform studies that involve drug intervention we perform pilot studies in a small number of animals to determine the most appropriate dose of drug to use for the larger study, thus minimising the need to study multiple doses, which would need more experimental groups.

All of our studies are designed to ensure that we obtain as much information as possible from each animal; this includes animal characteristics (phenotype), changes in physiology (such as blood pressure), alterations in blood biochemistry (such as blood cholesterol) and tissue structures. Where there may be tissues/organs that are not required for a particular study (e.g. blood vessels) we try to use these for isolated tissue experiments that will provide pilot data for future studies. Any other tissues are retained in a biobank, details of which will be available in the Institutional repository, in the event that they may be of use at some point in the future by ourselves of other academic groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Some animals will be given novel drugs for a period of time; in the majority of cases this will be achieved by adding the drugs to their food or drinking water, but in some cases (<5% of animals) where this is not possible (for example palatability problems that cannot be overcome by the use of treats) we may need to administer drugs by a small pump implanted under the skin. This procedure results in transient moderate discomfort that is managed by the use of appropriate pain relief.

We will also determine the glucose status of some animals, by giving an injection of either insulin or glucose followed by blood sampling; the technique used for blood sampling from the tail is similar to that used by individuals with diabetes who measure their own blood glucose by finger prick.

Why can’t you use animals that are less sentient?

At the end of the diet period any further investigations are performed under terminal anaesthesia (i.e. the animals do not regain consciousness). For some studies, where relevant genetic strains exist, we employ the C. elegans roundworm to perform in vivo studies, but although they have a close genetic profile to humans, their physiology is very different from mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have already incorporated as many refinements as possible to minimise the mild impact of the dietary interventions in our animals; animals are group housed and provided with environmental enrichment. Any animals undergoing surgical implantation of pumps for drug delivery are given peri- operative pain relief. For animals that are tested for fasting glucose status we minimise the period of time required for fasting and use a method of blood sampling that causes only mild and very transient discomfort.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We refer to the NC3R's guidance on experimental design and follow their best practice approaches for refining our experimental methods.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Project Licence holder attends local meetings with other Project Licensees to learn and present about 3R approaches in different research groups, and the Establishment senior animal technician is part of a regional group that meets to discuss refinements in animal husbandry etc; both of these networks are a valuable source of information and inform us at to what improvements to make within our own establishment. The Establishment also has a NC3Rs/ARRIVE committee that meets 2-3 times per annum to discuss recent advances in the 3R's and agree on the implementation of changes; this committee also scrutinises external grant proposals for projects involving animals to ensure that the 3R's and ARRIVE guidelines are adhered to in the experimental design.

179. Genetic basis of convergent evolution in guppies

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Evolution, genomics, guppy, life history, quantitative genetics

Animal types	Life stages
Poecilia reticulata	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to uncover the genetic basis of traits that are known to have evolved repeatedly in wild guppy populations. We will use a breeding cross approach to map genes to phenotypes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Convergent evolution is the process by which independent populations evolve similar traits in response to common environments. Classic examples include wing evolution in bats and birds. Convergent evolution can occur within species as well and a classic example of this is the the Trinidadian guppy.
Here independent guppy populations have repeatedly evolved to different predation levels, evolving different life histories and morphologies depending on whether they are in high or low predation environments. However, very little is known about the genetic basis of these adaptive traits.

To fully understand evolution and the adaptation process we need to determine the genetic basis of traits. By understanding the genetic basis involved in convergent

evolution, we can begin to understand the limitations of evolution, for example does the interaction between genes on traits limit the number of possible traits? Does selection on genes differ between the sexes? By answering these questions we can more fully understand what limits evolution. To answer these questions we need a well-studied example of convergent evolution with genomic resources. Therefore we are using the Trinidadian guppy.

What outputs do you think you will see at the end of this project?

Through this project we will uncover the genetic basis of convergently evolving, adaptive traits in guppies, specifically female and male life histories and morphological traits. By understanding the genetic basis of these traits we can understand why evolution tends to repeat itself over and over again. For example, do genes interact to produce a single trait? Do the sexes use different genes for similar traits? Are different traits underpinned by the same genes? By answering these questions we can understand what limits evolution and why some traits and trait combinations repeatedly evolve. These are fundamental questions in evolutionary biology.

The benefit is primarily basic research. However, future applied work can benefit from this work as well. For example, life history (timing of age and size at maturity) is an important consideration for maximising output in farmed fish. The genetic basis of adaptations and the limitations of evolution, can help researchers predict the ability of species to adapt to new environments (i.e. climate change) or why species are in decline.

However, for many species we lack both the genomic resources and the in-depth understanding of selection on phenotypes. As well, there are ethical considerations of studying species of conservation importance. Therefore, this research makes use of well-studied system that is easily kept in the lab, the Trinidadian guppy, to fill these gaps.

Who or what will benefit from these outputs, and how?

The primary benefits will be academic. This work will further the study of convergent evolution by being the first to map genes to convergently evolution traits in the Trinidadian guppy.

How will you look to maximise the outputs of this work?

We have already begun collaborations with other researchers interested in the evolution of traits but lack genomic resources and expertise found in my lab, including research on experimental evolution, sex chromosome evolution, and the evolution of colour. I will also attend at least 2 international and 2 national conferences during the course of the license to disseminate this research.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The guppy is a model system for evolutionary biology, with unparalleled information on how phenotypes evolve in the wild. However, the genetic basis of this evolution is largely unknown. We are focussing on evolution of life history traits, e.g. size and age at maturity. Therefore we will be using juvenile to adult guppies.

Typically, what will be done to an animal used in your project?

To measure life history of males, males will be housed individually for a maximum of 5 weeks (average 2 weeks), (step 1 of protocol 1) and given recovery anaesthetic to accurately measure age at maturity. Maturity in male guppies is defined as when the gonopodium, the anal fin that has been modified to become an intromitten organ in guppies, has completely developed (step 2 of protocol 1).

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect the fish to experience stress associated with the protocol (less than 5%). Adverse effects and stress will be recognised by loss of body condition, change of colour, clamped fins, behavioural changes including erratic behaviours or lethargy/hiding, no feeding response, and increased time surfacing.

Expected severity categories and the 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 maximum of 600 male guppies will be subjected to protocol 1, which has a maximum 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?

The project takes advantage of the model system, the Trinidadian guppy. While our lab has uncovered genes under selection in wild populations, they do not link genes to traits. This experiment therefore is an important piece of the puzzle for understanding evolution in this system, and is not possible without the use of live animals. The proposed work is essential to advancing knowledge in the field of evolutionary biology and is not possible without the use of live animals. The Trinidadian guppy system is unparalleled in its knowledge of natural selection on complex traits and therefore ideal to address these questions.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives would not be able to answer the main question of the project; linking genes to traits. We have, however, used simulations and results from past studies to show that easily detected genes underlying adaptive traits are likely in this system. Therefore, this project is likely to successfully map genes to traits in this system.

Why were they not suitable?

The above simulations and previous studies cannot directly link genes to traits and therefore determine whether convergent evolution at the phenotype level equates to convergent evolution at the genetic level. To do so we need an animal system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This project is designed to capture the population-wide genetic variation that affects adaptive traits in guppies, by analysing multiple families linked by shared grandparent fish. The proposed sample sizes were determined from computer simulations of multiple family experimental designs.

Over the duration of the project 600 male and 600 female from the second generation (F2) of the breeding cross fish are required for the analysis. We will initially cross 12 males to 24 females in grandparent (F0) crosses, resulting in 240 offspring (F1 parent fish, i.e. 120 full-sibling crosses). The resulting offspring (F2) will be processed as outlined in the project plan, with only the 600 male fish being subject to a licenced protocol. Grandparent (F0) and parent (F1) fish will be preserved for DNA analysis only. In order to produce that minimum number of second generation fish (F2), we first assumed that the average brood size of a virgin female guppy is 10, and then calculated the number of F1 and F0 crosses needed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In designing this experiment we have taken advantage of recent advances in quantitative genetics breeding design in order to minimise the number of animals we will need to use in this project whilst at the same time ensuring adequate reliability and reproducibility. For example, using the multi-family cross design has halved the number of grand-parental male samples required as 1 male is used for 2 separate crosses. We will use males and females from the same crosses, therefore minimising the number of broods (and surplus fish) required.

We will use the terminally sampled animals for multiple endpoint measurements in order to maximise the data from each fish and in doing so minimise the overall numbers needed, i.e. gut length, body and head morphology, and finally for DNA analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use the minimum possible number of animals of each sex in order to produce the results required to gain meaningful inferences from this research and to answer the objectives laid out. As well as the experimental design features mentioned, the number of animals required has been guided by our previous experience maintaining and using guppies as a model species.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Trinidadian guppies are model systems for studying selection in life history both in the field and aquarium settings. Measuring life history phenotypes in the lab are well established and have been designed to minimise risk of suffering. All protocols have been refined to ensure that the maximum possible stress experienced by the fish is mild. We will measure both male and female life history but only male measurements require a licenced procedure. Maturity in males will be assayed by measuring gonopodium development (the gonopodium is a modified anal fin in male guppies). Individual males will be visualised using a dissecting microscope under recovery anaesthesia, as outlined in Protocol 1. This method has been refined in close collaboration with long-established labs using guppies as a model system. Additionally the process has been streamlined so as to minimise the amount of time that the fish is under anaesthesia and can be completed in approximately 30 seconds in a moist environment. This will only be undertaken by staff who are suitably trained and deemed competent.

Life history of males will be standardised by individual rearing. Maturing male guppies will need to be housed individually to ensure standard diet and social history. However, by using clear plastic between tanks and porous barriers within tanks, each individual will be in visual and chemical contact with others (i.e. the same water source throughout the tank). Guppies are routinely housed in this manner with no adverse effects reported.

Why can’t you use animals that are less sentient?

As the model species in evolution, the use of guppies is fundamental to this project. We are not able to use more immature life stages as we are specifically measuring life history, that is, the age and size at maturity.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Lone rearing: Stress resulting from isolation will be minimised by providing chemical and

visual contact with other fish. They will be housed individually for a maximum time of 5 weeks: from the age of 5 weeks, as from our own observations, and previous data, this is the earliest age at which maturity may be seen to a maximum of 10 weeks.

Recover anaesthetic: Care will be taken to ensure that the correct dose of anaesthetic is used in accordance with the establishment's standard protocol. The fish will be carefully observed whilst in the anaesthetic solution and will be removed as soon as the fish has ceased swimming and lost the response to touch. After imaging under a microscope, fish will be returned to a well-aerated recovery bath of clean water and observed for signs of recovery (jaw and opercular movements). Only trained and competent staff will carry out the procedures detailed. The procedures used are optimised so the male guppies will be subjected to anaesthesia for a minimal amount of time and minimal number of repeats to ensure accurate data.

Non recovery anaesthetic: Any male fish that are mature, or have gone through their maximum 7 anaesthesia's, or have not reached maturity by 10 weeks of age can all be euthanised immediately post gonopodium assessment without recovery in between. This is possible due to the speed with which we can perform our assessments of maturation (typically 30 seconds) and is a refinement of our methods which will help reduce suffering in those animals.

All stock and other experimental fish will be housed so as to minimise stress at a density of between 2 - 7 per 1.5L tank, with provision of appropriate environmental enrichment (simulated gravel on the base of the tank and plants to provide a refuge).

All fish will be monitored daily for stress and any adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will work in line with relevant Standard Operating Procedures used in the establishment as well as taking guidance from husbandry and methods in previously published work in this field. Health checks for general wellness and for signs of stress will be carried out daily by trained technicians.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep up to date with the latest advice and information on the 3Rs by accessing relevant webinars and events via the NC3Rs website and the e-newsletter. Additionally I will keep in regular contact with the Named Persons and technicians within our establishment. If the opportunity arises I will aim to review and implement any of our experimental approaches.

180. Immunotherapy of cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immunotherapy, Cancer, Immunosenesence, Vaccination

Animal types	Life stages
Mice	adult, pregnant, neonate, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aims of this project are:

To develop new treatment strategies to stimulate immune responses against cancer including developing new protocols that rely on the patient’s immune system to prevent or treat cancers.

To minimise and prevent adverse effects of treatment strategies while maintaining their anti-cancer effect.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The results will increase our understanding of how cancer interacts with the immune system and thus, we will be able to optimise the experimental strategies, in order to efficiently treat cancer. In addition, we will obtain data regarding the safety and efficacy of

these strategies that are required to initiate clinical trials in cancer patients.

Findings will also be published in widely read journals to spread the knowledge to the scientific community.

What outputs do you think you will see at the end of this project?

The results will increase our understanding of how cancer interacts with the immune system and thus, we will be able to optimize the experimental strategies, in order to efficiently treat cancer. In addition, we will obtain data regarding the safety and efficacy of these strategies that are required to initiate clinical trials in cancer patients.

Findings will be presented at national and international conferences and will also be published in widely read journals to communicate the knowledge to the scientific community.

Who or what will benefit from these outputs, and how?

Results obtained will increase our understanding of the mechanisms involved in interactions between cancer and the immune system so that effective strategies for induction of immune mediated elimination of cancer cells can be developed. Findings will be published in peer-reviewed journals to disseminate the knowledge to the scientific community. These data will be used by other groups world wide for further studies, thus increasing our knowledge of the immunotherapies for cancer and the complications arising from treatment of some cancers. One of these complications is graft versus host disease (GVHD). Following treatment for leukaemias, or blood cancers, where the cancer cells are eliminated, many patients need to receive replacement, healthy immune cells, a transplant. These healthy immune cells can attack the patient's own tissues and organs resulting in graft versus host disease (GVHD). In severe cases, if left untreated, GVHD may prove fatal. It is therefore critical to treat and reduce the effects of this complication. There will be long term benefits as the data is assimilated and acted upon by the scientific and medical communities.

How will you look to maximise the outputs of this work?

We strive to publish all the results we obtain from our studies in peer reviewed publications which are available world wide. We also have an extensive network of collaborations both with members of the scientific community in several institutions both in Britain and abroad as well as with Pharma companies investigating cancer immunotherapies.

Species and numbers of animals expected to be used

- Mice: 5,100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice make efficient research animals because their anatomy, physiology, immune

responses and genetics have been extensively studied and so have become well-understood by researchers. Studies in mice demonstrate that many of the immune responses exhibited by mice are remarkably similar to those exhibited by humans and so results from these studies can form the basis for developing treatments for humans. Results published by other researchers allow us to build on the accumulated data world wide and so progress medical and scientific investigations more rapidly. We will be using adult mice, some of which are genetically altered which can therefore serve as excellent models for research into normal and cancer cells.

Typically, what will be done to an animal used in your project?

In a typical experiment, a mouse will be injected intravenously with tumour cells. This tumour will have a fluorescent marker on it so the tumour can be tracked. The mouse will be imaged to confirmed engraftment of the tumour. The mouse will undergo treatment for the cancer e.g by adoptive transfer of CAR-T cells with anti tumour properties by injection of the Car-T cells intravenously. The efficacy of this treatment will be monitored by imaging the mouse twice a week. This whole process may take several weeks (8-12). The number of procedures a mouse will undergo will be kept to a minimum. Mice which have been treated for tumour and have recovered will be rested possibly for a few months and may be re-challenged with tumour. This is particularly relevant for vaccinated mice. To confirm the vaccination has been successful, it is important to show that the tumour either does not return, or the kinetics of tumour re-growth has been delayed as a result of induction of host immune responses. Mice will be humanely killed at any time if they develop adverse clinical signs.

What are the expected impacts and/or adverse effects for the animals during your project?

We plan to inject tumour cells under the skin on the back of mice in order to induce cancer, with the aim to test if our therapeutic strategies can control tumour growth and if they can be well tolerated. Tumour growth will be monitored by imaging mice (expected to cause only transient, minor discomfort) and graft versus host disease by analysis of immune cells from blood sampling from a peripheral vein.

Anticipated adverse effects, caused by cancer and/or graft versus host disease, include loss of appetite, weight loss, hunching, ruffled fur, lethargy, hind limb weakness.

Treatment of mice for tumour growth is expected to reduce tumour size and so is expected to reduce the adverse effects experienced by the mice. Additional efforts will also be made to minimise these adverse effects by providing extra bedding and tissues for cages, heat boxes, warming cabinets and wet mash diets to make the mice more comfortable. The vet and animal care staff will be consulted with regards to analgesics for the mice.

Weight loss will be monitored and weight loss of 10% of pre-treatment weight will result in daily monitoring. Mice which experience weight loss of 20% of their pre-treatment weight will be humanely culled. Mice developing adverse effects giving cause for concern during the course of the study will be humanely culled immediately. All remaining animals will be humanely culled at the end of the study and tissues will be taken for analysis and further study to inform us on the treatment outcome.

Mice inoculated with solid tumour inducing cells will be monitored regularly to avoid distress to the mice. Calliper measurements will be carried out typically 2-3 times in a 7day

period. Daily measurements will be performed when the mean cumulative diameter of tumours reach 10mm. When assessing the characteristics of injected tumours, the mean cumulative diameter will be limited to 12mm. For tumours under therapeutic treatment, the mean cumulative diameter of tumours present will be limited to 15mm. At any stage, mice with tumours experiencing more than mild discomfort 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)?

In all cases, the adverse effects are not expected to be more than moderate in severity. It is anticipated that tumour will be established in 95-100% of mice. The growth of the tumour may lead to weight loss, ruffled fur, lethargy, hunching, hind limb weakness. With treatment, some mice may experience only mild symptoms and moderate symptoms may be experienced by 75-90% of mice.

Weights will be monitored and mice will humanely culled at any time if they are found to have lost 15% of their starting weight in the presence of other clinical signs or, 20% of their pre-treatment weight in the absence of other clinical signs.

(In our experiments, we work to guidelines contained within 'Guidelines for the welfare and use of animals in cancer research'. By P Workman et al; British Journal of Cancer (2010) 102, 1555 – 1577.)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Given the complexity of immune interactions with cancer cells, indications of potential safety/efficacy issues obtained from non-animal studies in culture dishes will need to be evaluated in living animal models. When living animal studies are required, they will be designed to mimic the human clinical setting as far as possible so that translationally relevant results are obtained from these living animal studies.

Which non-animal alternatives did you consider for use in this project?

We have developed non-animal culture dish models of immune and cancer cells that allows us to study many aspects of their interactions without needing animal-based studies. This enables us to identify how the various components of the therapy work together. Accordingly, animal experimentation will be undertaken only after culture dish studies have demonstrated potential safety and efficacy of an immunotherapeutic strategy.

Why were they not suitable?

Many aspects in our development of safe and effective immunotherapies will be adequately addressed by laboratory-based investigations, without requiring animal studies. Immunotherapies will only progress to whole animal studies if favourable results are first obtained from the laboratory based culture dish experiments. As these therapies will progress to clinical studies it is essential they are evaluated in animal models first. Laboratory based, culture dish experiments can only test a few of the possible interactions between the various cell populations at a given time. These studies therefore cannot fully represent all the numerous interactions possible within the whole body. In order to ensure the safety and efficacy of any treatment options, the culture dish treatment options need to be tested in a model as close to the whole human body as possible so any adverse effects will be picked up and any safety and efficacy issues addressed before the treatments are considered for human treatment. The mouse model provides such vehicle for these pre-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?

Estimation of the numbers of animals we will use have come from a number of sources. Statistical analysis of previous experimental results have been used to estimate the numbers we will require in these experiments to ensure we use the minimum numbers of mice but also ensuring the data we produce is statistically sound so that experiments do not need to be repeated unnecessarily.
Experiments from other labs using the same animal models available in peer reviewed journals have also provided useful data on which to base our estimation of the animals we will need to generate statistically relevant data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To assure use of minimum animal numbers, small-scale preliminary experiments, known as pilot studies, have been carried out to determine the minimal numbers of mice required for statistically significant and reliable results. This will help minimise the total number of experiments required. We have incorporated pilot studies into all the experimental plans in order to reduce required numbers of mice during experiments.

We have frozen sperm from genetically altered mice which are not required for immediate use. To have mice ready for experiments, the mice need to be bred and a colony maintained even when no immediate experiments are planned. In order to reduce the constant production of mice, sperm from genetically altered mice have been frozen so that mice are generated by fertilising eggs and the resulting embryos implanted into female mice, generating mice for experiments only when required.

This total numbers of mice has increased slightly as there are now a greater number of independent projects on this licence compared to our previous licence.

We are mindful of the need to keep our animal usage to a minimum and these numbers reflect our efforts to strive to use the minimum numbers possible.

In an effort to keep our numbers low, we have and will continue to consult a number of publications and online tools in experimental design and reduction e.g

NC3Rs. Online advice resource portal. https://www.nc3rs.org.uk/topic-specific-resources-0

The AWERB "Guiding principles on good practice for Animal Welfare and Ethical Review Bodies" 3rd Edition – September 2015.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Small-scale preliminary experiments will reduce the number of animals required and we have included animals for pilot studies in our protocols. In addition, only experiments that are likely to be relevant for use in human clinical studies will be employed.

We have reduced our breeding numbers considerably and intend to breed only when necessary and to keep this to a minimum.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 engineered mouse models for our studies. Cells derived from mouse tumours will be injected into genetically identical hosts followed by treatment to eradicate the tumour. Cancer models using cells from different species will also be utilised to examine the immune responses against human tumour cells. Human leukaemic or lymphoma cells will be injected into immunocompromised mice and these tumour cells will then be treated in an attempt to eradicate the tumour. Tumours which induce solid tumours when transplanted will also be injected. The results obtained using mouse cancer models are already accepted as being indicative of responses in humans. Small-scale preliminary experiments, 'pilot studies', will reduce the number of animals required. In addition, only experiments that are likely to be relevant to use in human clinical studies will be employed. Animals will be monitored closely and will be humanely culled before they develop signs of distress.

Why can’t you use animals that are less sentient?

We need to use animals which closely represent the human immune condition. The in vivo studies will be designed to mimic the human clinical setting as far as possible so that translationally relevant results are obtained from animal studies. We need to use living animals that are representative of the complex interactions that occur between body

systems. The adult mouse provides such a model for our experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All our animal experiments will be performed using mice, some of which may be genetically modified. Tumour growth will be closely monitored using similar technology to that applied to imaging patients with cancer, thus ensuring minimal discomfort to mice.

Imaging

Imaging is not usually associated with any adverse effects however if any effects are seen e.g. redness of skin, mice will be treated as advised by the vet.

Anaesthesia

Light anaesthesia by inhalation will be used where possible during imaging to allow restraint. Depth of anaesthesia will be monitored regularly and animals receiving inhalation anaesthesia delivered by air will be allowed to recover fully before being returned to their home cage. No adverse effects are expected from the use of light anaesthesia and mice generally recover very rapidly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The AWERB "Guiding principles on good practice for Animal Welfare and Ethical Review Bodies"3rd Edition – September 2015

NC3Rs. Provide online resources and guidelines for 3Rs. (E.g guidelines on humane endpoints and welfare assessments. https://www.nc3rs.org.uk/topic-specific-resources-0

Workman et al; British Journal of Cancer (2010) 102, 1555 – 1577; Guidelines for the welfare and use of animals in cancer research.

Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

The above are publications which we have made use of, are readily available online and as hard copies and which provide guidance and advice on best practice experimental use of animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3Rs is an online regular publication which covers a wide range of topics and provides advice and information on replacement, refinement and reduction in animal research. This publication will be consulted and advances within the field will be taken note of.

181. Investigating the (Patho) Physiological Importance of Protein Modifications

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

intellectual disability, neuronal communication, neuronal development, protein function

Animal types	Life stages
Mice	neonate, juvenile, adult, aged, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the function of specific proteins in the brain and nervous system and their links with disease. The proteins of interest are enzymes that modify other proteins in the brain and nervous system and have been implicated in disorders including intellectual disability and neurodegeneration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Intellectual disability is a condition affecting up to 1% of the general population and involves a defect in intellectual and adaptive skills. This condition can be caused by gene mutations, environmental insults or injury, and there are no available drug treatments or cures. Neurodegeneration typically occurs in later life and involves the loss of function and death of neurons in the brain (common neurodegenerative disorders include Alzheimer's disease, Huntington's disease and Parkinson's disease). There is also no cure for these conditions.

It is important to undertake this work because the proteins being investigated have been linked to neurological disorders, including intellectual disability and neurodegeneration. Intellectual disability is characterized by below-average intelligence or mental ability and a lack of skills necessary for day-to- day living, whereas neurodegeneration refers to the progressive loss of function and death of neuronal cells in the brain. By studying the functions of these proteins we hope to generate new insight into the changes that occur in the nervous system in these and other neurological conditions, which will lead to the identification of potential new 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 molecular and cellular changes that occur in the brain in intellectual disability and other neurological disorders. 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 journal and conference presentations and posters.

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 function of related proteins. The data we report will provide new ideas and insight to advance their studies. In addition, the research will also benefit clinical researchers who are investigating the underlying causes of intellectual disability, neurodegeneration and related disorders- 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 or 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 as our work 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 that make up the cells of an organism) to ensure that we develop a detailed understanding of molecular changes that are caused by deficits in our proteins of interest, and which may be linked to intellectual dysfunction and neurodegeneration.
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 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 (notably chemistry) and seek to develop new methodologies and chemical tools to maximise our scientific endeavours.

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: 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 are using mouse models to investigate the underlying causes of intellectual disability. 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 neurological disorders. Indeed, the main mouse model used in this work has been shown by our group and others to exhibit many of the key features seen in patients who have mutations in the affected gene. Mice are typically used from post-natal day 1 through ~3 months of age, as we are investigating changes in brain structure and function, the temporal nature of these changes, and the behavioural consequences of these brain changes in older mice (10-12 weeks age).

Typically, what will be done to an animal used in your project?

Typically, genetically altered (GA) mice are culled by a humane schedule 1 procedure and brain tissue removed for analysis. In addition, some mice are used in a range of behavioural tests that may run for a duration of several weeks. After behavioural/physiological measurements are completed, mice will be humanely culled and, if appropriate, tissue may be taken for further studies.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse characteristics that we expect to observe in our GA mouse strains are only of a Mild nature, including deficits in learning and memory, sociability etc. We have certainly confirmed this for the main mouse model that will be used in this project and do not expect to observe other clinical signs as a result of other genetic manipulations. However, as we may be using novel genetically-altered mouse lines, we cannot state this with absolute certainty. There is expertise within the animal care staff in developing scoring systems that, if necessary, can be used to ensure that no animal experiences a severity level beyond that of Mild. Where appropriate, we will develop and use these systems in consultation with the NVS.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most (estimated 80%) expected to be Mild; some (estimated 20%) expected to be sub-threshold.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal usage is essential to allow us to examine the effects of disrupting specific genes on the function of the nervous system and in development of disease. Genetically-altered mouse strains are the most frequently used animal model to study the importance of specific genes/proteins for physiology and to provide appropriate disease models, and their use in the current project is essential. Where possible, we will complement these studies by 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?

We will use mammalian cell lines wherever possible to limit animal usage. However animals are required where it is important to understand the function of a protein in neurons or other cell types in the brain or within neuronal networks or specific brain regions- these analyses cannot be undertaken in cell lines. We have also considered the use of lower organisms but it is not possible to study more complex brain behaviours relevant to the neuronal disorders relevant to our work (e.g. intellectual disability, deficits in movement of the mouth and tongue that might be linked to speech problems) in these other model systems.

Why were they not suitable?

Whilst analysis of cell lines can provide (and will provide) important complementary information, it is not possible to study higher integrated processes such as learning and memory using cell-based assays, and simple model organisms (e.g. yeast) lack the required complexity and physiological pathways to allow a detailed understanding of protein function 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 our current mouse model over

the previous two-year period. I anticipate that similar numbers of these mice will be used over the 5-year period of the project licence (~300 per year) and have included an additional 1,000 mice (with modifications of different genes) that 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?

We take several steps in the experimental design phase to reduce the number of animals being used. This begins with the breeding strategy that we have implemented, which is designed to ensure that all male mice can be used in experimental analyses and that the majority of female mice can be used as breeders, thus minimising wastage. This breeding strategy also allows us to compare mutant offspring with their non-mutant (i.e. normal) siblings, which limits experimental variability and therefore reduces the number of animals required to generate robust 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 the same animals in multiple 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 mice, 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 predominantly be using a knockout mouse model (a type of specially bred mouse that has a specific gene disrupted) that we have been studying for the last five years. These mice show no obvious signs of distress, weight loss or reduction in life-span. The experiments we are undertaking are either minimally invasive behavioural measurements or extraction of tissue following schedule 1 killing. These methodologies are designed to minimise any distress to the animals.

Why can’t you use animals that are less sentient?

Analysis of complex behaviours such as learning and memory, anxiety etc and analysis of development/dysfunction of the nervous system cannot be done using less sentient species. The mouse models used provide the most suitable species to understand how specific proteins and gene mutations function in humans (and how they can be targeted in disease). We cannot used terminally anaesthetised animals in behavioural assays and

where experiments are performed using tissue, the animals are humanely culled before tissue collection.

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 Mild 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. Animals in behavioural tests will receive treats and habituation to the tests in order to reduce any potential stress.

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

182. Phenotyping Genetically Altered Mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Phenotype, Mice, Pleiotropy, High-throughput, Genetic Altered Animal

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?

To phenotype genetically altered (GA) mouse strains including determining the pleiotropic effects of genes and gene mutations.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is estimated that the function of over 50% of genes is either poorly understood or not known. Although studies of Mendelian disease loci, genome-wide association studies (GWAS) and genome sequencing studies have uncovered a large and very valuable dataset on the relationship between gene and phenotype in humans, a recent study has also shown that a quarter of all research has been done on only 0.5% of known human genes1.

Between 5,000 - 8,000 rare diseases are thought to affect the human population, and 80% of these have been identified as having a genetic origin. Although each disease may affect only 0.1% of the population, together the lives of 3 million people are impacted. This is partly due to the dramatic acceleration of human population over the last 5,000 years

where an excess of rare variants has occurred within the human genome but without sufficient time to allow for natural selection2. One key element of understanding how genetic variations cause rare diseases is being able to isolate the relevant mutations in the human genome that are primarily responsible. Given the genetic variation within the human population, isolating the causative mutation is difficult. Whole exome sequencing (WES) has enabled the scientific community to sequence only the exome, or protein-coding, part of the genome. This exome sequence makes up just 1 - 2% of the human genome and is a much cheaper and less data-heavy method than whole genome sequencing. As a result, isolating the potential genetic mutations that cause rare diseases has become easier as familial and non-familial genomes can also be studied alongside that of the patient.

Through genetic manipulation, other mammalian models can be used to mirror genetic changes in human patient groups and thereby examine cause and effect. One of the most important scientific tools in understanding mammalian gene function is the laboratory mouse which shares around 98% of its genes with humans, as well as similar physiology and anatomy for the majority of the organ systems. An extensive toolkit for the manipulation of the mouse genome and the generation of new disease models exists including use of mouse ES cells and genome editing techniques such as CRISPR/Cas9.

In order to address the challenge of determining the function of all the genes in the human and mouse genomes, or the consequences of genetic variation, there are many large and smaller scale functional genomics projects underway. Some of these involve the generation of a wider and more sophisticated collection of mouse mutant strains. For example, the principal aim of a largescale worldwide phenotyping project (LSPP) we are involved in is to generate and phenotype mutants for every gene in the mouse genome, providing an important baseline for gene function in the mammalian genome and underpinning fundamental new insights into biology and the genetic bases of disease. This has built upon, but importantly does not overlap, other large-scale phenotyping efforts. Although our participation in this phenotyping project is likely to finish in 2021, the wealth of resources and technological developments that we have generated will serve well as a basis for moving from lines with null mutations to answering specific clinical and scientific questions using lines with point mutations. In addition, a peer reviewed and funded genome editing programme we have been running for several years is, now on its 7th iteration, this enables researchers to apply for relevant genes of interest to be designed and created at our facility with either null deletions or point mutations.

Other disease-focussed efforts for which mouse strains are being generated investigate more specific, sometimes more complex (oligogenic) pathologies.

Other large-scale projects include ENCODE, the Encyclopedia of DNA Elements. This project has recently published its findings from Phase 3, expanding the database of not only DNA elements in the human genome but also providing further resources on the DNA elements in the mouse genome3. This led to the development of a freely accessible database (screen.encodeproject.org) further enabling the use of mouse genetics in understanding human diseases.

Technological advances are also leading to ever more sophisticated, accurate and refined mouse phenotyping. This progress inevitably is accompanied with the requirement for extensive validation, ongoing and constant quality control and rigorous equipment maintenance. The phenotyping service that we offer includes access to advanced phenotyping equipment and procedures in fields of study such as metabolism,

neurobehaviour, chronic pain and imaging. In addition, there is a current need and demand from the UK scientific community for extensive genetically altered (GA) mouse phenotyping.

Researchers come to us to access equipment and expertise outside of their own specialist fields which may be important in the interpretation of their own results revealing novel gene function and ensuring that confounders to future research are known before strains are used extensively. For example, a mutant mouse with impaired sight might not be suitable for tests relying on a reaction to a visual cue. To this end, the mouse lines we receive are accompanied with welfare and husbandry information. We can then design bespoke phenotyping pipelines based on line information and scientific need/justification, breed and manage experimental groups throughout phenotyping testing and disseminate the data back to the scientific community appropriately.

Dolgin, E. (2017) The greatest hits of the human genome. Nature 551:427-431

Tennessen JA et al. (2012) Evolution and functional impact of rare coding variation from deep sequencing of human exomes. Science 337: 64 - 69

ENCODE Project Consortium (2020) Expanded encyclopaedias of DNA elements in the human and mouse genomes. Nature 583: 699

What outputs do you think you will see at the end of this project?

We anticipate developing and characterising up to 300 GA lines as part of the LSPP and as service work. Of these, we expect two-thirds of these lines to be characterised through high-throughput phenotyping screens and 100 of these to be characterised in depth. New GA lines created here are done so under a different project licence. As part of that licence, each line will have gone through a full validation process, including extensive sequencing, to ensure that the GA is fully characterised.

Phenotyping data collected for the LSPP will continue to be published not just by the LSPP but also utilised in publications throughout the scientific community to inform other research. Welfare data will also be collected and distributed with mice when they are exported.

As part of the service work, we expect to provide data and samples to external clients to further inform their research resulting in publications and continued scientific research.

Who or what will benefit from these outputs, and how?
Primarily the wider scientific community will benefit from both the LSPP data and the phenotyping service that we can provide. We anticipate that not only basic research will benefit but also clinical research and other large-scale projects such as Genomics England 100k Genome. Data from pre- clinical models will be fed back directly to clinicians and patient groups and this data could be utilised in drug-testing on another licence.

How will you look to maximise the outputs of this work?

The LSPP maintains a publicly available web portal allowing access to all LSPP data, metadata, protocols and enabling the scientific community to request GA lines and tissue. We ensure that all lines generated as part of the LSPP are freely available either through the European Mutant Mouse Archive (EMMA) or through one of the other key repositories

around the world.

Issues that impact on large-scale high-throughput phenotyping or are likely to have an impact on specific models and refinements to techniques will be disseminated through publication.

All raw data will be disseminated to clients for their use and we anticipate that this will be then used in publications. Our phenotyping service will be advertised either on site to external visitors, as part of our new training facility, or publicised at UK conferences and meetings alongside other services that we offer.

Species and numbers of animals expected to be used

- Mice: 60,000

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.

Whilst it is now possible to carry out genetic manipulations in frogs and fish, the processes being studied here are being studied in a mammalian context and, although other animal and non-animal species can be informative in this regard, they cannot supplant studies specifically in mammals. Mice remain the model of choice due to the wealth of the genomics information available, their relevance to humans and the relative ease of generating, establishing and cryopreserving mouse colonies. Moreover, well-developed and characterised inbred strains of mice present an opportunity for reducing variability and therefore enhancing reproducibility.

We are studying neonatal, juvenile, adult and aged mice due to the unknown nature of the GA lines that will be tested. To study these different characteristics it is necessary to study the mice at various life stages, from birth, through to adulthood and ageing. Where GA mice are not viable we may study embryos to help us understand why the mice cannot survive without a particular gene or set of genes.
Typically, what will be done to an animal used in your project?

Many of the mice on this licence will be used only for breeding. This is due to the need to carry out several steps of breeding to obtain a suitably sized cohort that can be studied. We will also maintain breeding lines to ship to collaborators for use on other projects authorised to accept GA lines.

In terms of phenotyping testing, approximately 25,000 mice will go through one of four protocols. Some GA lines will be initially assessed on Protocol 3 for suitability for phenotyping pipeline studies. GA lines will then go through a broad-based phenotyping pipeline, a bespoke phenotyping pipeline that drills down into specific phenotypes and a small subset of lines will go through a short pipeline for studying pain response. At a specific age point (e.g. early adult or late adult) may involve up to 12-15 tests, most of which are non-invasive and a small number involve anaesthesia used for immobilisation only. Mice are then finally anaesthetised and a terminal bleed carried out. All pipelines are expected to end by 78 weeks of age.

What are the expected impacts and/or adverse effects for the animals during your project?

There are many possible adverse effects that could occur due to genetic modifications of the mouse genome. Moreover in a number of mouse strains we will be breeding the genetic alteration to homozygosity for the first time. Possible adverse phenotypes are those that lead to sub viability such that homozygotes generally die between birth and weaning. Other adverse effects that might occur during the lifetime of the mice include deterioration in health, tremors and lack of condition.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

5,700 mice on the mild breeding protocol are not expected to suffer any adverse effects and the vast majority will not reach higher than a sub-threshold severity.

On the moderate breeding protocol, it is anticipated that of the GA lines being bred to homozygosity for the first time, 50% could exhibit a moderate phenotype, approximately 27,000 mice.

All 23, 600 mice on Protocols 3, 4 and 5 are expected to reach a moderate severity. This is partly due to the phenotype of the mice, in which the genetic alteration could lead to a moderate severity in around 50% of the mice (the other 50% being unaffected controls). Secondly the pipeline of phenotyping tests the mice will undergo is likely to lead to moderate suffering, although this will be short term. Whilst most of the tests are mild there are one or two that are likely to push the severity into the moderate category, such as fear conditioning. Additionally, the combination of mild tests may lead to a cumulative moderate suffering. However, all mice will reach a moderate severity due to a small set of the phenotyping tests causing moderate suffering, for example electroretinograph (ERG). Whilst these moderate affects will be short lasting they will increase the maximum severity of all animals on this protocol to moderate.

Of the 600 mice on the pain pipeline of Protocol 6, 100% will reach a moderate severity due to the formalin test.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For the studies performed in this licence it is not possible to use non-animal alternatives. Every phenotyping assay requires the complex interplay of multiple organ systems only

apparent in a whole organism such as the mouse. For example, when examining the effect of a potassium channel knockout, it is important to understand the impact of this deletion which may affect neurobehaviour, cardiac function or another system. For many lines, we will be studying multiple systems involving many organs of the body, and the interplay between them (e.g. behaviour and metabolism). There is currently no cellular system in which we could presently hope to make a comprehensive study of the pathological processes involved in causing disease or the underpinning biological pathways.

For other GA lines, the design of the phenotyping pipeline will be driven by the questions being asked by external research groups. In these instances, we will request evidence from the research group with regards to replacement strategies that have been investigated. This could typically be from the PPL used to initially establish the GA lines but might also derive from peer-reviewed processes such as grant applications. Furthermore, we have established a strong international network of collaborators over the years and we now have access to a rich source of information across many different fields in science as well as in husbandry and welfare. We utilise this network for areas of research that lie outside of our own expertise, to ensure that we are able to identify areas of work where either the mouse model or the phenotyping test is not appropriate to the scientific question. It is further hoped that this project may contribute tools to replacement research in the form of tissue for cell lines or data for computer modelling.

Where we are conducting service work, ensuring that grants funding the service work have been peer reviewed will be an essential requirement prior to undertaking the work.

Which non-animal alternatives did you consider for use in this project?
No non-animal alternatives were considered for this project.

For the majority of service work, we anticipate that previous work will have been done by the researcher in either tissue culture or human studies to inform whether a mouse model is viable for further research. For example, previous experience has shown us that the hypotheses behind a lot of the service work has come about from either studies with other animal models such as fly models, from clinicians working with patient groups or from stem cell work.

For LSPP, the remit is to understand genes in a mammalian context therefore it is essential to focus on mammalian models rather than non-mammalian models.

Why were they not suitable?

In order to understand the pleiotropic effect of a GA model, it is necessary to study the global impact of a gene knockout on multiple systems working in concert. An animal model is currently the only way to understand these global effects.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The LSPP phenotyping pipeline has been designed by the entire LSPP consortium and has involved the input of many laboratory animal experts. By utilising such a pipeline we maximise the data obtained from each cohort of mice. Combining tests in the same mice allows the interpretation of data to be correlated directly, rather than inferred. This allows us to carry out more advanced statistical analysis to detect correlated trends between assays. Directly correlated effects have a greater sensitivity as the major source of variance (between mice) is removed, opening the possibility to detect subtle effects resulting from gene deletion, thus resulting in more data from fewer mice. The principles and statistical tools used in the LSPP are extensively used for other bespoke phenotyping projects.

For the LSPP we use a cohort sample size of 14, consisting of 7 males and 7 females. Under the most powerful design-analysis combination, if we increased the sample size from 14 mice to 20 mice, we would improve the chance of seeing a phenotype by 15%. However, if we decreased the sample size from 14 mice down to 8 mice, there would be a 31% decrease in the likelihood of seeing a phenotype. Therefore a sample size of 14 mice is calculated to be the optimum, with very little gained by increasing the sample size.

For non-LSPP projects which include tests that are not conducted as part of LSPP, we will base the numbers required for a phenotyping cohort on the test within the pipeline that has the most variance. For tests that lie outside of the LSPP remit, the number of animals used will be based on power calculations using previous baseline data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The LSPP is a major international effort and therefore has access to many resources including full-time statisticians from different centres who form a permanent working group which discusses refining and advancing the statistical models used which include:

Phenstat: High-throughput phenotyping generates large volumes of varied data including both categorical and continuous data. Operational and cost constraints can lead to a work-flow that precludes traditional analysis methods. Furthermore, for a high-throughput environment, a robust automated statistical pipeline that alleviates manual intervention is required. PhenStat is a package that provides statistical methods for the identification of abnormal phenotypes with an emphasis on high- throughput dataflows. The package contains dataset checks and data cleaning prior to the analysis. This statistical package has been tested and demonstrated using 420 lines of historic mouse phenotyping data from the Sanger Mouse Genome Project and Europhenome resources (Karp et al., PLoS Biol., 2014).

Bayesian Statistics: Bayesian linear and logistic multilevel regression models are also used (De Angelis et al., Nature Gen., 2015). Metadata for each phenotyping test that are considered potentially relevant by domain experts are collected to include in the overall analysis. This includes sex, strain, litter, day of analysis and other experimental metadata such as equipment used and certain details of the procedure, for example how the blood samples were handled. Changes that can occur in the baseline data over time and the effects from day of analysis and litter were also taken into account to enable the correlation of phenotypes among groups of mice. Using this method, phenotypic differences can then be determined between mutant and wild type animals. Significance thresholds are then chosen to control the false discovery rate at 5%.

For tests conducted that are not part of the LSPP remit, a local bioinformatician is involved calculating sample sizes based on both the pipeline that will be used and taking into account previous data. Online power equation calculators are used to look at individual tests.

Standard Operating Procedure's have been written and used routinely for previous projects. This standardises the way the data is collected and reduces the variability and therefore the sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All breeding for experimental cohorts is performed according to precise breeding calculations, the formulae of which we refine and update periodically with reference to current breeding figures.

Numbers of mice used for GA breeding is frequently reviewed and altered breeding schemes are trialled to maximise productivity and minimise overbreeding.

All crosses use dynamic mating systems, where the male is removed prior to littering thereby avoiding second litters and the risk of overbreeding.

Breeding figures are examined by the local Animal Welfare and Ethical Review Board (AWERB) on an annual basis for all the service licences which includes generation and phenotyping of GA lines.

Mice for phenotyping are typically bred by heterozygous intercross with the homozygotes and wild types then being phenotyped. The heterozygotes produced are used for embryology, archiving, supply for research groups and expression studies.

When a gene knockout as part of a publicly-available project results in mice that are of immediate interest to the scientific community, we inform the relevant domain experts before the mice reach the end of the pipeline in order to utilize as many tissues as possible without having to breed more mice.

Where breeding information is not known (i.e. for new lines) a small pilot breed will be carried out first to assess viability. These mice, if viable, can be used for the first cohort of the study, and remaining larger breeds will be set up taking into account information gathered from the pilot breed.

All animals will be randomly assigned to cages and technicians and data analysts will be blinded to genotype until the study is completely finished and data analysis complete.

Part of theLSPP remit is to generate a tissue biobank that is accessible to the public. We are committed to this and we maintain a tissue biobank of lines generated for utilisation by the research community.
This ensures further data dissemination without the need to breed additional mice.

We are also committed to providing new GA lines of high quality. All new lines generated go through an extensive QC process and genotyping is designed specific to each allele. New GA lines are generated under the authority of a different project licence and once

validated, are then bred under the authority of 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.

For LSPP, it has already been defined that null knockouts will be used on a C57BL/6NTac background.

For non-LSPP projects, we will be guided by the scientist as to which is the best mouse model to use to understand the scientific question being asked. However we will have discussions on baseline strains and controls to ensure we are satisfied that all factors have been considered.

Test	Why is this the most refined method?
Ear biopsy	Mice need to be ear clipped for identification and the same piece of tissue is used for genotyping. Genotyping protocols have been optimised to use such small samples for all types of genotyping that we currently carry out. This is more refined than other methods such as a tail biopsy.
Induction of transgene expression	In most cases this will be done by oral gavage on five consecutive days. Previous data has shown that this number of doses is necessary for sufficient induction of expression. This is more refined than an injection, as whilst it involves restraint there should be no pain. Injections may be necessary depending on the type of recombinase line used and the time at which it needs to be activated.
SHIRPA	SHIRPA is an observational test that involves placing the mouse in an arena or a jar and looking for abnormalities. This is non-invasive and can provide a lot of phenotypic information that can be followed up in further, more complex tests. This is also a good opportunity to pick up any more subtle welfare concerns.
Click box	This test is non-invasive and involves an observation of movement of the ears in response to a high-pitched tone played close to the mouse. This provides a good opportunity to assess the hearing of a mouse before going on to more in-depth ABR studies and can inform other tests such as fear conditioning if the mice are known to be deaf.
Behavioural Observations	This test is a non-invasive, observational test whereby mice are placed either in an arena or are observed in their home-cage. This can provide a lot of phenotypic information that can be followed up in further, more complex behavioural tests.

Open field	Open field gives us data on the reaction of the mouse to a novel environment. This arena is anxiety inducing in that the mouse has not seen it before and the light is relatively bright. There are no smells or sounds that should cause any further stress to the animal.
Grip strength	The grip strength test is the quickest, least invasive way of measuring muscle strength. The test lasts for less than one minute and should cause no pain, only stress induced by handling.
Rotarod	Rotarod is useful to measure activity when the mouse is encouraged to run by placement on a moving rod. It is also a useful cross laboratory comparison to ensure reproducible phenotypes. Where possible, we will use voluntary wheel running to assess co-ordination or motor function as there is less stress induced from handling and the animal can choose whether to use the wheel or not.
Gait analysis	This involves placing the mouse in an arena and videoing for a short time. This is the most refined method of running this test as, unlike some other gait analysis equipment, it does not involve any stimulation to force the mouse to move.
Acoustic startle and pre-pulse inhibition	For this test it is necessary to assess the response of the mouse to an audible stimulus. Whilst the stimulus itself is anxiety inducing, efforts are made to reduce stress by having a constant background noise and carrying out the test in the dark. A protocol has been developed over many years to give useful data over as short a time as possible.
Spontaneous alternation	This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are set so that the mouse can see but are not intended to by anxiety inducing.
Elevated plus/zero	This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are anxiety inducing in some areas of the arena but are darker and not anxiety inducing in other areas. This is necessary as the test assesses the preference of the mouse to the darker or lighter areas.
Social recognition	This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are low to reduce anxiety. In this test another mouse is present in a section of the arena. This mouse is in a small cage to stop any aggression but the scent and sight of it is needed to assess behaviour of the test mouse to a social stimulus.
Novel object recognition	This test is now carried out in a home cage environment. This reduces the stress on the animal and the novel object may be seen as a form of enrichment.
Temperature taking	This is done using a rectal probe that should cause no more than transient discomfort. The animal is only lightly restrained and the test is done in the shortest time possible, usually less than one minute.
Urine collection	Mice are held and urine caught in a container, or in some cases mice are placed on a plate and allowed to explore while urine is collected below. In both cases this is less stressful than placing the mouse in a metabolic cage and collecting urine overnight.
Light dark box	This is non-invasive and involves placing a mouse in an arena and allowing it to explore. Light levels are anxiety inducing in some parts of the arena but not others. This is necessary as the test assesses the preference of the mouse to the darker or lighter areas.

Ophthalmoscope	For this test the mouse is held whilst the eyes examined. This is more refined than more in depth imaging as it does not involve general anaesthetic. However, the data collected is more subjective and there may be times when in-depth imaging is needed, in which case mice will undergo optical coherence tomography (imaging of the eye under general anaesthesia).
Optokinetic Drum	This test involves the mouse being placed on a platform in a large enclosed environment under low lighting levels. The mouse is completely surrounded by monitors which display black and white lines at various widths and moving at various speeds. This assesses the visual response to movement stimuli and adds to the phenotypic information garnered in other eye tests.
ECHO-MRI	This test involves less approximately one minute of light restraint whilst body composition is measured. This is more refined than alternative tests which require general anaesthesia and a longer time to gather the data.
Blood sampling	Blood samples are collected up to a maximum of 15% total blood volume of the animal, which has shown no adverse effects in previous studies. Samples are taken from the tail vein using a very small cut and mice have local anaesthetic applied to the area twenty minutes before the bleed takes place.
Tolerance tests	Blood samples are collected up to a maximum of 15% total blood volume. The fasting period for this test is usually 18 hours due to the need to allow animals to use up glucose supplies and enter gluconeogensis. However, where possible fasting times will be reduced (e.g. 6 hours maximum for insulin tolerance). This test is more refined than other methods of measuring glucose which require surgery to implant indwelling catheters.
Video tracking for sleep	This is non-invasive and measured in the home cage, however due to recording equipment animals have to be singly housed.
Circadian	This is non-invasive and measured in the home cage, however due to recording equipment animals have to be singly housed.
Voluntary Wheel Running	This is non-invasive and measured in the home cage, however due to recording equipment animals have to be singly housed. This is a refinement on the rotarod test as animals can choose whether to run on the wheels.
Standard calorimetry	This is non-invasive in home cage-like equipment. Bedding is provided and animal shelters are used when activity measurements are not needed. This test is used to measure oxygen and carbon dioxide levels which are used to calculate metabolic parameters.

Advanced calorimetry	This is non-invasive in home cage-like equipment, however the floor is gridded and no shelters or bedding are available. As this is more stressful than standard calorimetry it will only be used when it is essential to have parameters that can only be measured with this equipment. This test is used to measure oxygen and carbon dioxide levels which are used to calculate metabolic parameters. In this equipment it is also possible to alter the temperature and lighting regimes, as well as measure body weight and food weight.
Metabolic cages	Mice are housed for as short a time as possible, usually 20 hours. This length of time is needed to collect a sufficient amount of urine for further analysis and to assess overnight food and water consumption. Red houses are placed in the cages to provide shelter and respite from the grid floor.
Home cage monitoring	This is non-invasive and measured in the home cage. After initial insertion of a microchip this test involves no further pain, suffering or distress to the mouse. This generates large amounts of data with no adverse welfare effects. The main parameter measured is activity but videos may be used to assess other behaviours.
Stress induced hyperthermia	This test is necessary to assess stress without relying on activity. Mice will be closely monitored throughout and protocols have been designed for the shortest time period possible. At all times mice are in a home cage but will be singly housed and with limited bedding as it is necessary for this to be a stress inducing environment.
Cold test	Mice are placed in a cooled environment (approximately 4 degrees centigrade) for up to 6 hours. Mice are closely monitored and removed if the temperature drops too low. This test is used to assess how well the mouse can regulate it's own body temperature.
Hot plate	This test has been refined so the mice are removed from the apparatus at the first sign of a response to the heat. Two technicians and multiple mirrors are utilised to ensure the first response is not missed. This test assesses the thermal pain response of the mouse.
Von Frey	This involves putting pressure on the mouse paw with a filament. The number of filament presentations has been reduced in this method as legacy data on mice shows the need to focus on a particular size of filaments. Mice are habituated to the arenas to reduce stress. This test assesses the mechanical pain response of the mouse.
DEXA/X-ray	DEXA and X-ray are now done using one piece of equipment which has been refined to allow constant observation and the use of gaseous anaesthetic. Both tests can be done together to reduce the need for further anaesthesia. This test measures bone mineral content and density, as well as producing an image to assess bone structure and abnormalities.
Echo-ultrasound, Electrocardiogram	These tests are done under gaseous anaesthesia. When mice require both tests they are carried out under one anaesthetic. Potential skin damage from shaving is mitigated by the use of hair removal cream. These tests either image the heart to measure structural abnormalities or produce a read-out of the electrical activity of the heart.

Auditory brainstem response	This test is done under general anaesthetic due to the extremely sensitive nature of the measuring electrodes and the need for a completely sealed sound proof box. Mice are monitored at all times through a window and the electrodes themselves should cause no pain. This test measures the electrical response of the brain when specific sounds are presented.
Pupillometry	This test measures the pupillary responses to light to measure the non-visual functions of the eye. This test is non-invasive but does require the adjustment to a dark environment and the exposure to a bright light.
Remove Adhesive Fine Motor Test	This test measures the fine motor co-ordination of a mouse. Mice are gently restrained and a small sticker applied to the forehead. Latency to initiate removal and total removal time are scored.
Optical Coherence Tomography	This test is done under general anaesthetic to ensure that the mouse is immobilised for the duration of the test. A small camera is positioned close to the eye and images are captured of the back and the front of the eye.
Fear Conditioning	This test assesses the ability of mice to learn and to assess their contextual memory and their cue memory. Mice are placed in chambers and subjected to a short electric shock to the paws after a sound tone. This test has been refined from using three electric shocks to using one electric shock. The mice are placed into the chamber again the next day to assess whether they associate the environment and/or the sound tone with the fear response initially induced by the electric shock.
Electroretinograph	This test is done under general anaesthesia due to the extremely sensitive nature of the measuring electrodes and the need for contact lenses to be held over the eyes of the mouse. This test measures the electrical responses of the eye to light stimuli. The electrodes are fine enough to not cause any pain.
Formalin Test	This test has been refined so that the injection of formalin into the hindpaw is done under general inhalation anaesthesia. The mice are then placed in an open arena with mirrored walls and are constantly monitored for the following 60 minutes as well as video-recorded. The mice are culled immediately following the test. Pain responses are then counted from the video recording. This assesses the chemical pain response of the mouse.
Operant Test (touch screen)	In this test the mouse learns a series of pictures on a computer screen and receives a food reward for correct answers. It can be used to test motivation and is less stressful for mice than other motivation tests, such as seeing how long the mouse will swim for before giving up.
Hypoxia Challenge	This non-invasive test measures the respiration of mice when the air content is altered to 10% Oxygen and 3% Carbon Dioxide for a period of five minutes. The test is repeated on a second day with the mice placed in different chambers to account for variability and the data is an average of the results from the two days.

Why can't you use animals that are less sentient?

The mouse is the lowest mammalian species (in terms of neurophysiological sensitivity) in which the full range of genetic manipulations can be achieved necessary for the scientific

projects this licence services. It is also possible to carry out some manipulations in frogs and fish but the processes being studied here are being studied in a mammalian context and, although other animal and non-animal species can be informative in this regard, they cannot supplant studies specifically in mammals. Mice remain the model of choice due to the wealth of the genomics information available, their relevance to humans and the relative ease of generating, establishing and cryopreserving mouse colonies.

Moreover, well-developed and characterised inbred strains of mice present an opportunity for reducing variability and therefore enhancing reproducibility.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all tests it is important that the animal has no additional stress, therefore mice are handled calmly and habituated to testing rooms as well as arenas if possible.For all tests mice are only housed in modified cages or arenas for the minimum time needed to gather meaningful data. Mice undergoing phenotyping tests are monitored more frequently and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the phenotyping tests. Mice which have had anaesthesia have extra monitoring until fully recovered and extra checks when back in the holding rooms. When general anaesthetics are necessary, the combinations with least adverse effects will be used, for example for all tests inhalation anaesthetics will be used, with the exceptions of the Auditory Brainstem Response, the Electroretinograph and the Optical Coherence Tomography test which cannot be carried out with the mouse on a face mask. Pain from tail bleeds is reduced by using local anaesthesia.

This licence involves no surgery.

Pipelines are designed with thought given to the overall experience of the mouse and the number of type of tests any one animal will go through.

This licence will also run in parallel with a Phenotyping Development licence and once phenotyping tests have been validated, procedures will be refined in tandem.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from LASA guidelines.

The animal house has full AAALAC and ISO (9001:2015) accreditation. To conform to these standards we must ensure a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses and can be found at https://www.mousephenotype.org/impress

ARRIVE guidelines will be followed at all times.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Project leads will attend general 3Rs symposiums in the UK and abroad over the course of this project. From these we may gather information on refined phenotyping techniques or housing and husbandry methods. Moreover, it is our intention to continue to present any techniques that we develop ourselves in posters and papers in the relevant scientific/animal care forums.

More specifically, members of the phenotyping team will attend LSPP conferences that focus on all aspects of the project and the pipeline. Any new developments which could impact the LSPP studies will be discussed within technical working groups and brought before a scientific advisory board.

183. Rodent models of pulmonary hypertension and associated co-morbidities

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Pulmonary hypertension, Pulmonary arterial hypertension, Pulmonary vascular remodelling, Right heart failure, Treatments

Animal types	Life stages
Mice	adult, juvenile, pregnant
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To evaluate the role of identified molecules (gene and proteins) and miRNAs in rodent models of pulmonary hypertension and pulmonary artery banding models.

To test agents for their ability to prevent progression / induce regression of pulmonary vascular disease and cardiac function.

To develop novel rodent models of pulmonary vascular disease and associated co-morbidities based on our better understanding of disease drivers and pathogenesis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Pulmonary hypertension (PH) is caused by elevated blood pressure in the vessels carrying blood to the lungs. It is categorised into several groups, depending on the underlying cause. Pulmonary arterial hypertension (PAH) is a rare but fatal disease with approximately 8500 prevalent cases in the UK. PAH is caused by the tightening of the blood vessels in the lung causing a progressive narrowing and eventual blockage due to cellular growth (much like cancer), leading to strain on the right side of the heart, ultimately leading to heart failure. Current treatments for PAH focus on alleviating symptoms by targeting the vascular constriction but have no effect on cellular growth within the vessel wall.

Furthermore, no treatments specifically target the right side of the heart. Our research focuses on understanding the mechanisms regulating the remodelling processes within the lung blood vessels and the consequences on heart function. By understanding these mechanisms, we aim to identify the key regulators and potential drug targets.

What outputs do you think you will see at the end of this project?

We are continually generating new knowledge about the disease processes and progression of Pulmonary Hypertension. We have already identified several potential new mechanisms, novel drug targets, and new treatments to modulate pulmonary hypertension. This project will allow us to develop a greater understanding of how these new drug targets (and drugs) modulate disease by testing them in these models and provide further key data to either halt, or promote studies geared toward clinical translation.

Who or what will benefit from these outputs, and how?

The short-term impact of this work includes the publication of research manuscripts and provision of data to commercial partners to support the progression (or halting) of drug candidates. The time scale of these varies from study to study but generally from 6 months to 2 years after the completion of the studies conducted under this license. Longer-term impact can arise from the clinical translation of any drugs or procedures towards clinical trial or hopefully routine clinical use. The time frame for this impact also varies greatly but may be in the region of 2-15 years after the completion of studies performed under this license.

How will you look to maximise the outputs of this work?

We collaborate extensively with academic and industry collaborators to ensure we

maximise the impact of each study through both publication, and where possible through driving clinical translation. In addition to the publication of full research manuscripts we also actively present our research at the main medical and scientific conferences with our field.

Species and numbers of animals expected to be used

- Mice: 11300 over 5 years
- Rats: 1000 over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Pulmonary arterial hypertension is a rare but devastating condition that can present in all life-stages from the Newborn to later life. Most frequently the disease manifests in early to mid-adult life, and can occur as a disease in it's own right, or as a common cardiovascular co-morbidity to other lung and heart diseases. Tissue samples are extremely limited and only available from patients with end-stage pulmonary hypertension when undergoing lung transplantation (rare), or at post-mortem. Animal studies are therefore necessary to study disease pathogenesis and the role that various environmental and genetic factors play in the progression of disease. Adult mice and rats are the smallest mammals available that possess a cardiovascular system that is sufficiently similar to human, that can be non-invasively and invasively measured to allow the study of the underlying disease processes and assess the impact of treatments.

Typically, what will be done to an animal used in your project?

Typically, we will induce pulmonary hypertension (mostly commonly by a sub-cutaneous injection of a drug, often in the combination with exposure to low oxygen) to ascertain whether loss/blockade of a gene/proteins protects against/reduces the development of disease over the period of 3 (mice) to 9 (rats) weeks. Since this license is underpinned by research with a strong translational drive, a large proportion of experiments will involve administering agents that we hope will alleviate disease (most commonly via injection or orally) and therefore symptoms and suffering. In mice where we are studying disease mechanisms, some animals may be irradiated and undergo a bone marrow transplant to elucidate the contribution of a specific gene in inflammatory cells (or tissue) to disease. To assess the disease progression, and/or determine the efficacy of any treatments some animals may require the implantation of telemetry devices to allow for the continuous measurement of blood pressure and /or undergo non-invasive imaging. In both cases anaesthesia will be required, most commonly inhaled isoflurane which is very rarely associated with any adverse effects. Our procedures end with an invasive cardiac catheterisation to measure pressure in the chambers of the heart under terminal anaesthesia, followed by tissue harvest. On average, the typical animal will undergo 5 procedures. 1) Non-invasive baseline imaging; 2) Disease induction; 3) mid-point pre-treatment imaging; 4) treatment; 5) Final end-point imaging, cardiac catheterisation and tissue harvest.

What are the expected impacts and/or adverse effects for the animals during your project?

The most common adverse effect will be associated with the development of pulmonary hypertension. Regardless of the model used, and much like human disease, these present as a general malaise, loss of appetite, breathlessness and weight loss. Models of PH typically induce disease over a period of 21 days for mice, and between 3-6 weeks for rats. Mouse studies are typically 6 weeks (3 weeks for disease to develop and 3 weeks to perform treatment). Rat studies are most commonly 9 weeks in duration, 3-6 weeks for disease to develop and 3 weeks to perform treatment). Changes in animal welfare as disease progresses are generally subtle in the first instance and slowly progressive. On occasion, a small number of animals can develop a more rapid disease progression and weight loss.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity of these studies is moderate for both mice and rats. Rarely, animals undergoing surgery may incur surgical complications in which case they will be humanely killed. All animals will be humanely killed at the end of the study, or if they are deemed to be likely to exceed moderate severity limits.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 function of the lungs at the most basic level is to facility exchange of oxygen and carbon dioxide from the blood to the breathed air. This process is impaired in pulmonary hypertension. Pulmonary hypertension arises from the complex and dynamic interaction between multiple cell types in multiple organs that regulate the flow of blood to the lungs, and the subsequent gas exchange processes. It is not currently possible to model all these aspects in silico or in vitro (including in isolated organoids and lung slices) as they lack the ability to fully reproduce the interaction of air and blood within a functioning heart-lung system. The use of rodent models is therefore essential to allow us to study the biology of the cardiopulmonary system as disease develops, and ultimately test whether we can impact on these processes through deletion or blockade of specific molecules and assess the efficacy of drug treatment.

Which non-animal alternatives did you consider for use in this project?

We consider, and indeed use a combination of human tissues where available through collaboration. We also utilise blood samples collected from patients locally to identify molecules of interest, however, as stated above these are only available from patients with advanced disease. We have considered the use of ex-vivo organ culture models, zebrafish

models, and in vitro cell experiments using human and rodent commercial cells but ultimately these only provide sufficient data to support rather than replace the need to conduct the experiments and procedures outlined in this license.

Why were they not suitable?

None of the alternatives described above provide a complete system to study the interaction of all the cells, tissues and organs affected by pulmonary hypertension (heart and lung), and the complex process of gas exchange from breathed air and blood. We need this complete system in order to understand the interaction between these different cells and molecules in disease, and study the impact of modulating identified molecules on other tissues and organs ie to identify potential side effects of a future therapy.

In vitro approaches lack sufficient complexity to study multiple cell types in a familiar environment - e.g. cells growth on plastic in vitro 'behave' very differently than when maintained in vivo. Organoids or tissues slices similarly lack the ability to study the complex interaction between blood and air, and again are often performed in a very different environment to that of the whole animal system. As such these models only a short time frame where the organoid or tissue are of use. Zebrafish can provide a model for some basic studies but the gill structure used for gas exchange under water is obviously different to the mammalian air breathing system. The zebrafish is also a single blood circulation from a 2-chamber heart which is in contrast to the 4-chamber mammalian system with the left chambers pumping and oxygenated blood around the body and the right chambers pumping de-oxygenated blood around the lungs.

Although we utilise these systems, and each play its part we do require an animal model that can reproduce the human disease at the level of blood vessels, the heart and their interaction with blood and the multitude of inflammatory cells types contained within it, and the air we breathe, as closely as possible.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These figures are based on multiple studies conducted over previous licenses. We typically use experimental group sizes of 8-10 mice or rats, with each experiment in the region of 50 animals depending on the number of treatment groups to be compared. In any given year we typically perform 3- 4 such rat, and 3-4 mouse studies. The establishment and maintenance of mouse lines and hetero/homozygous (dependent on line) breeding of cre-lox transgenic colonies (currently over 20 lines) to generate the procedural animals with tissue specific gene knock-out typically accounts for 2000 mice per year. These numbers allow us to test the role of 4-6 genes/drugs per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use a combination of cells and patient material to perform a large number of studies to examine expression and function of molecules in in vitro models, prior to initiating any in vivo studies.

We also take a multi-methodology approach to phenotyping the rodent on procedures to ensure we capture non-invasive (where possible), accurate and longitudinal data. This reduces error and variability between animals thereby reducing experimental group sizes. These protocols allow us to select in the relevant steps required for each study, similar to planning a procedure using the NC3Rs EDA, ensuring we use as few animals as possible for each study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have optimised the number of breeding mice, and timed mating within each line to generate the required numbers of offspring for experimental procedures. We are currently exploring the use of intermittent breeding strategies to reduce the number of offspring not required for experimental 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.

Mice and rats are the lowest form of species that have a ‘humanlike’ cardiopulmonary system. The models proposed are widely used throughout the scientific community and we are recognised as being one of the best labs at performing them. Mice represent the best model to dissect the role of the gene in disease development due to the increasing availability of ‘floxed’ knockout mice. Rats represent the best rodent model in terms of disease complexity and are therefore the model of choice to perform therapeutic studies on established disease.

All rodents are monitored daily and are assessed within the severity limits set out within this license. Animal health and welfare is assessed using a scoring sheet, which evaluates body condition scoring, behaviour and aspects of the mouse/rat grimace scales. Adverse effects are written up on protocols and monitored. All animals will be humanely killed at the end of the study, or if they are deemed to be likely to experience effects of more than moderate severity.

We work closely with the Named Veterinary Surgeon and animal care staff to ensure that the procedures and animal husbandry conditions are as refined as possible. For example, we perform handling training and offer high value rewards (e.g. condensed milk or banana chips) after oral gavage, high calorie feed to offset weight loss in diseased rodents. We also combine procedural steps e.g. treatments with blood sample to measure drug levels where possible to minimise stress, and offset with high value ‘treats’. We utilise a species

specific animal welfare scoring system to monitor disease progression and sign post to humane endpoints. In addition, we aim to utilise rat tickling in future studies to minimise the stress caused from handling and procedures, such as oral gavage and i.p. injection. This technique mimics natural play habits and is recognised by NC3Rs as a refinement to improve rat welfare.

Why can't you use animals that are less sentient?

To fully study the disease processes in humans we require an already developed (adult) mammalian cardiopulmonary system that can be 'easily' manipulated to study the functional role of molecules in disease development. The nature of the disease requires multiple techniques to diagnose humans with the disease, and we employ similar methods in rodents. We use the smallest available pressure catheters to measure heart pressures in mice so are limited by size in smaller, or younger animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We closely monitor all our animals and have set criteria based on appearance, behaviour, malaise and weight loss. As pulmonary hypertension develops in mice and rats, they do display signs of malaise and breathlessness, which are used as a sign that the disease model is developing. We have developed a welfare scoring sheet to formalise these criteria and record our daily checks of the animals. The scoring sheet will enable daily monitoring of welfare as disease develops and progresses. This will ensure any deterioration will be captured at the earliest opportunity and highlight animals for further monitoring before any rodent exceeds our severity limit. Weight loss remains an important indicator of welfare. Where any mouse or rat records a loss of >10% either from their starting weight, or from their previously recorded weight, twice daily checks using the scoring sheet and daily weights are recorded until the rodent gains weight on 2 consecutive days (returning to daily checks and twice weekly weights). Any mouse or rat that records an additional 10% weight loss in the next 24h will trigger a humane endpoint. We have an experienced team of researchers with 5-15 years' experience running these procedures and initiate feeding of high calorie treats (trail mix) to help maintain body weight and reduce welfare costs in these models.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will utilise general best practise guidelines covering the general use of laboratory animals such as those provided by NC3R's e.g. Resource Hubs including Experimental Design Assistant, ARRIVE guidelines etc and the procedureswithcare.org.uk website.

For best practise specific to the use of cardiovascular and pulmonary hypertension model we will additionally refer to:

Bellantuono I, de Cabo R, Ehninger D, Di Germanio C, Lawrie A, Miller J, Mitchell SJ, Navas- Enamorado I, Potter PK, Tchkonia T, Trejo JL, Lamming DW. A toolbox for the longitudinal assessment of healthspan in aging mice. Nat Protoc. 2020;15:540–574.

Bonnet S, Provencher S, Guignabert C, Perros F, Boucherat O, Schermuly RT, Hassoun PM, Rabinovitch M, Nicolls MR, Humbert M. Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension. Am J Respir Crit Care Med. 2017;195:583–595.

Lawrie A. A report on the use of animal models and phenotyping methods in pulmonary hypertension research. Pulm Circ. 2014;4:2–9.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We commonly share best practice with both industry and academic collaborators though conferences and research meetings. We actively keep abreast of research publications in our area and look for ways to implement any improvements that we can see for example consulting with the NC3Rs regional programme manager and NC3Rs website to gain up-to-date knowledge.

Home Office

184. Using zebrafish to model skeletal disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Zebrafish, Bone, Cartilage, Joints, Disease

Animal types	Life stages
Zebra fish	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?

To advance understanding of the role of genetics, environmental factors and loading in the development, repair and degeneration of skeletal structure.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Most people will suffer from a painful skeletal condition at some point in their life. In the UK over 15% of the population suffer from chronic back pain and over 50% will develop arthritis, in at least one joint, by the time they reach retirement age. Over 50% of post-menopausal women and 25% of men over the age of fifty will suffer from osteoporosis which leads to bone fragility and a heightened risk of fractures.
Currently, few effective treatments are available for any of these conditions, all of which are projected to increase in incidence as the population ages.

We now understand that genetics and biomechanics (e.g. body movement) each contribute about 40% to the risk of developing skeletal disease, but how these genetic signals and mechanical stimuli influence the different cell types that make up the skeletal system remains largely unknown. The outlined work aims to advance fundamental

understanding of the processes that control skeletal cell function and thereby identify new gene targets that could be manipulated to improve outcomes in skeletal conditions. We also understand that obesity plays a role in skeletal disease, however separating the effects of loading from weight gain, from those caused by the presence of fat cells (adipocytes) or changes to the immune system are diifficult. As zebrafish are supported by the water we can study how changes to fat tissues affect the skeleton independent of loading.

What outputs do you think you will see at the end of this project?

The key output of these studies will be to advance understanding of the mechanisms underpinning normal musculoskeletal health and to identify how these alter in various disease states.

The study will focus on genes identified, in human population screens, as being involved in the development and maintenance of skeletal structures and investigate how these function within the body to influence the various cell types involved. Using this approach, we will identify and evaluate targets that could be used to develop beneficial therapies, e.g. that improve bone health or reduce joint inflammation. The study will also investigate the interplay between genes and the mechanical loading that the skeleton undergoes during development and various disease states. We will investigate how mechanical forces of compression and tension influence skeletal cell behaviour and what impact these changes have on the integrity of the skeleton or joints. We will also investigate how the immune system interacts with musculoskeletal tissues during injury and evaluate whether modulating the immune system could be used to improve bone and joint health. For each arm of this research our goal will be to publish our findings in top ranking, open access journals. We will also present our findings at scientific meetings, including those focused on the 3Rs.

Other tangible outputs will be new stable mutant lines (with mutations in genes associated with skeletal diseases) and transgenic reporter lines (which aid visualisation of skeletal development and regneration through non invasive imaging). Another output will be computational software to track groups of zebrafish during normal swim behaviour, and software to model biomechanoical loading of the skeletal.

Who or what will benefit from these outputs, and how?

In the short term, the findings of our study will benefit scientists working in the fields of skeletal biology, mechanics and genetics. In the medium term, the findings are expected to benefit biotech companies and big pharma in developing new products and interventions to improve skeletal and joint health. In the long term, the findings are expected to benefit clinicians and patients through the development of more effective treatments for common skeletal conditions such as osteoarthritis, osteoporosis, fracture repair and back pain.

Other researchers will benefit from the reporter and mutant lines that we will generate and share freely (we will ship them to the European zebrafish stock centre for non profit distribution). For example more than 60 groups worldwide use the cartilage and bone reporter lines generated under my previous project licences. When we characterise mutants, if we identify phenotypes of interest to other groups/disciplines we actively seek collaborations. For example, we generated a mutant line in a gene associated with osteoarthritis however, when it was subsequently learnt that the gene was also associated with hearing loss we gifted the line to a research group working on hearing.

How will you look to maximise the outputs of this work?

To maximise the output and impact of our research we will collaborate with clinicians specialising in gerontology, rheumatology and orthopaedics. In so doing, the direction of our research is guided by real and relevant clinical problems. We also collaborate widely with other scientists working in different, but complementary fields, such as engineers (biomechanics), mathematicians (modelling), cell biologists (genetics) and behavioural scientists (swim motion). These collaborations allow us to achieve more than we could alone by facilitating the adoption of complementary methodologies. Our work with clinicians allows us access to relevant patient information to improve the accuracy of our modelling.

As a group, we are proactive in science communication and have run events on the use of zebrafish in science to aid dialogue with the public about animal work and to promote the use of low sentient animal models, such as fish and flies. The findings of our research will be widely disseminated through presentations at international scientific meetings and by publications in high impact open access journals and review articles.

We will continue to gift our stable mutant and trangenic lines to the European stock centre for non profit distribution. We will use our 3Rs lines of communication (for example via the regional 3Rs manager) to help us to identify groups which could benefit from our computational software or from our Computed Tomomography data, thereby aiding others in reducing the numbers of animals they use.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 25650

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 adopts an integrated approach utilising computational modelling as well as in vitro, ex vivo and in vivo models. With respect to the latter, our studies will utilise zebrafish to investigate how the skeletal system forms and functions throughout life in response to changes in gene expression and the mechanical loading incurred during movement. A major advantage of using zebrafish for these studies is that their translucent bodies enables the use of live imaging to follow the processes of interest over relevant timeframes in a single animal and thereby reduces the number of animals needed to provide robust data. Where possible, in vivo studies will be undertaken using larval stages which are less sentient than adult fish. Nevertheless, adult fish are required for some studies, in particular in order to study the bones of the spine, which are not formed during larval stages. In some studies, we also use aged fish in order to study disease states associated with ageing such as osteoporosis and osteoarthritis.

Typically, what will be done to an animal used in your project?

The majority of animals required are only used for the creation and maintenance of lines

for breeding. Many of these fish are reporter lines in which specific cells are labelled with a fluorescent marker, thereby allowing internal processes to be monitored using non-invasive imaging. A relatively small proportion of the fish (approximately 10-20%) will be used in studies into the functioning of the skeletal system. Where possible these studies will be conducted using larval fish less than 5 days of age. Of the fish grown to adulthood, many will have their movement during swimming or feeding assessed using high speed video recordings. Some fish may be fed altered diets to assess if the addition or vitamins or changes to the amount of dietary protein or fat impact bone health. Some fish will be used in regeneration studies in which, following the induction of anaesthesia, either a small number of scales (which are naturally shed and replaced by the fish) are removed from the flank or a small number of the very thin bones of the caudal fin are fractured and the repair process subsequently monitored by non- invasive imaging. Some aged fish will be treated with clinically relevant drugs to assess how they work and why they are more effective in some people than others.

What are the expected impacts and/or adverse effects for the animals during your project?

The zebrafish used in regeneration studies are not expected to show any adverse signs, and are expected to continue to swim, feed and interact with other fish completely normally. Genetically altered fish carrying mutations in key genes relevant to skeletal development and maintenance, are expected to grow into adulthood and to continue to feed and interact with other fish although they may swim more slowly or have a 'lop sided' motion or show spine curvature. All fish are carefully monitored, and any showing signs of suffering are promptly 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 majority of fish (~80%) will only be used for breeding and will only undergo the mild or subthreshold severity procedures needed to determine their genotype. Approximately 12% of all fish will carry genetic alterations that could result in moderate suffering however, the vast majority of these are not expected to experience overt suffering. Fish entering the moderate severity regeneration protocols (~8%) will have a small injury inflicted under general anaesthesia e.g. amputation of a small section of tail fin, the removal of scales from a small area of the flank or the fracture of a small number of fin ray bones. Upon recovery from anaesthesia these fish are expected to immediately resume normal behaviour and to show no outward signs of suffering. Around 10% of fish will be used in ageing studies. These fish are not expected to show any obvious signs of suffering however, since the study involves imaging under anaesthesia and some fish are expected to develop spinal curvatures or fusions of their vertebrae (analogous to those seen in human ageing), they may experience joint stiffness and discomfort.

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 use of a vertebrate animal model is essential for studying the development and maintenance of the skeletal system in health and disease since these processes involve the interactions between far too many different cell types to be effectively modelled using either cell culture systems or computer simulation. Furthermore, it is not possible to undertake these studies in non-vertebrate models, such as the fruit flies or worms, as they do not have a comparable skeletal system. For these reasons, and because ultimately our experiments need to be clinically relevant, at least some of our experiments must be performed in a vertebrate species such as the zebrafish. Nevertheless, wherever possible the studies will be performed using embryonic or larval stages of development which are less sentient than adult fish.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, non-animal models will be used in preference to animal studies e.g. the effect of specific genes on mineralisation will initially be assessed in studies conducted in vitro by culturing cells carrying knockouts to various genes involved in skeletal development. In addition, computational modelling will be used to study the mechanics of the skeletal system. To this end we have already built the first Finite Element models (based on engineering paradigms) of the zebrafish jaw and spine to evaluate how alterations in bone shape and properties affect these structures. The data generated using this system has been the basis for a number of publications in peer reviewed journals. We are also, in collaboration with computer scientists working towards the development of a 'virtual joint' to model how cells respond to certain conditions, which will allow us to run some of our experiments computationally. Nevertheless, to validate the clinical relevance of the findings made using cell culture and computational modelling will still require the use of vertebrate models, such as the zebrafish.
Wherever possible, our fish studies will be performed using embryonic or larval stages of development which are less sentient than adult fish.

Why were they not suitable?

It is not possible to fully study the development and maintenance of the skeletal system in health and disease without using animal models as the processes involve are far too complex to be effectively modelled using either cell culture systems or computer simulation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 requested is based upon our estimate of the likely number of genes and cell types that will require manipulation in order to meet our objectives. In estimating the group size for the studies we have undertaken extensive analysis of data from our previous studies as well as drawing on data published by others and undertaken power calculations, with the help of G*power software and the National Centre for Replacement, reduction and refinement (NC3R)'s experimental design assistant (EDA). These calculations have been further checked by a specialist bio-statistician. The vast majority of animals requested will not be used in experimental studies but are required in order to breed animals with the genetic make-up needed for the experimental studies. Here again we have drawn on our own extensive experience and published information in estimating the number needed.

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 use the NC3Rs Experimental Design Assistant application to help in the planning and design of our experiments. We are also constantly alert to the possibility that effect size differences for new assays may be greater than expected, potentially enabling groups sizes to be reduced whilst still retaining the power to detect significance between experimental groups. Wherever possible, prior to undertaking regulated animal studies, preliminary investigations will be undertaken to test developing hypothesis and to assess the safety of novel compounds using our established cell culture models, computational models, or larval zebrafish. We have pioneered the use of ex vivo scale culture, which has enabled us to reduce the number of adult animals needed when assessing drug treatments by almost 100 fold. Each scale, of which a typical fish has 300-400 and which are naturally shed and replaced throughout the life of the fish, is a miniature flattened skeletal system. Wherever possible, harvested scales will be used to test putative skeletal drug prior to their use on adult fish.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The vast majority of fish will only be used to create fish with the correct genetic make-up for experimental studies. Care will be taken to ensure that all breeding programmes are undertaken with optimal efficiency. Wherever possible, we will coordinate breeding and ageing studies with colleagues working in related fields to maximise the usage of the animal produced e.g. by sharing clutches of transgenic fish amongst researchers using the same line/cross.

We will continue to develop and utilise computational modelling approaches to reduce the number of fish used in experimental studies by running many of our pilot studies 'in silico' i.e. on a computer to predict likely outcomes. This allows us to be more precise in our power calculations. We are developing AI approaches to better harness all available data from our imaging studies. To this end we currently have 2 full time researchers developing AI approaches, one producing programmes to automate the analysis of existing image data and the other developing a computational 'virtual joint' using engineering approaches to model how the cells of the skeletal system, grow, divide and mature in response to various stimuli.

We are also planning the tissue banking of samples collected from our aged fish at post-mortem, which we will be shared with other researcher groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish will be used to study the genetic basis of the complex interactions that occur between the cells involved in skeletal development and maintenance during health and disease. This will involve the generation of fish with genetic alterations affecting genes known to be involved in skeletal health. All procedures that could cause pain, suffering or distress to the animal will be performed under general anaesthesia. Upon recovery from anaesthesia the animals will be carefully monitored, and any showing more than transient signs of pain or distress will be killed.

The use of Zebrafish for studying skeletal repair and regeneration represent a significant refinement to the comparable procedure in rodents since, unlike rodents, the skeletal structures of the fish do not support the weight of the animal. There are more than 400 bone segments in the Zebrafish fin and following fracture of a small number of these, no discernible changes in the behaviour of the fish are observed.

In order to study how skeletal maintenance and repair alters with age some fish will only enter studies after they have been aged.

Why can’t you use animals that are less sentient?

Whilst a significant proportion of our studies will be conducted using zebrafish larvae; genetically altered adult fish are still required to generate these. We will also utilise a fish scale culture system that allows some experiments to be performed ex vivo rather than using adult fish, however these still require the use of genetically altered fish to provide the scales for the assay system. It is not possible to use non-vertebrate species, such as Drosophila or worms, for these studies, as they do not have a comparable skeletal system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures that could cause the animals pain, suffering or distress will be conducted under general anaesthesia. Upon recovery from anaesthesia any animal that shows more than transient signs of pain will be killed. In addition, we will be guided by colleagues within our scientific community (and beyond) with regards to best 3Rs practice for the procedures being undertaken. In particular, because the zebrafish is a relatively new animal model, we are continually in dialog with our research colleagues regarding innovative ways to refine our procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our zebrafish husbandry is in line with that recommended by FELASA and LASA. We follow the work and recommendations of zebrafish researchers on pain and experience and will continue to implement improvements wherever possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have a continuing dialogue with the NC3Rs and with other zebrafish researchers regionally, nationally and internationally. In recent years, I have presented talks at several NCR3s conferences about the use of zebrafish and computational modelling as substitutes for the use of rodent models. I constantly strive to develop models that either substitute or complement investigations normally performed in animals, including the computational modelling of skeletal structures.

185. Molecular mechanisms underlying neurogenesis and neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Nervous system, Neurodegeneration, Epilepsy, Signalling, Mitochondria

Animal types	Life stages
Mice	adult, pregnant, embryo, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to identify new genes and molecular mechanisms that regulate the generation of neurons during brain development and that regulate nerve function during nerve cell degeneration. The new genes and processes that are identified will then be targeted in models of neurological 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?

As a group, neurological diseases are the most complex and poorly understood. This project will elucidate the processes relevant to mitochondrial diseases and genetic diseases such as tuberous sclerosis complex, which cause epilepsy and autism. Discoveries from invertebrate models will underpin the proposed experiments providing a solid basis for achieving the aims. The project will benefit researchers in the fields of neurodevelopment and neurodegeneration by providing new knowledge that will help reveal how the brain develops and degenerates at the molecular level. In the long term this project will benefit patients with neurological disease and so society in general as it will

identify new molecular therapeutic targets and compounds that modify the disease process. Given the prevalence of diseases such as epilepsy, Alzheimer's disease and Parkinson's disease this project is highly worthwhile as it could have widespread impact.

What outputs do you think you will see at the end of this project?

The goal of this project is to identify new genes and molecular mechanisms that regulate the generation of neurons during brain development and that regulate neuronal dysfunction during neurodegeneration. In order to achieve this goal we will use mouse lines with specific genetic modifications that affect these processes.

Defects in neurogenesis lead to epilepsy, autism and other common neurological disorders. To elucidate the complexity of neurogenesis we need to identify key genes and molecules that regulate the generation and differentiation of neurons. This project will generate new knowledge that will lead to breakthroughs in the understanding and treatment of neurological disease.

Overall the project will identify important new mechanisms in brain development and disease. The main output will be in the form of publications in peer reviewed scientific journals. Other outputs will be the developmental of new animal models of neurological disease and new methods to study these models. In the long term this project will potentially lead to the development of new therapies to treat neurodevelopmental and neurodegenerative diseases.

Who or what will benefit from these outputs, and how?

As a group, neurological diseases are the most complex and poorly understood. This project will elucidate the mechanisms relevant to several of these diseases. The project will benefit researchers in the fields of neurodevelopment and neurodegeneration by providing new knowledge that will help reveal how the brain develops and degenerates at the molecular level. In the long term this project will benefit patients with neurological disease and so society in general as it will identify new molecular therapeutic targets and compounds that modify the disease process.

How will you look to maximise the outputs of this work?

We have several ongoing collaborations with other labs in the UK and USA. We will continue these collaborations and develop new collaborations during the project to generate synergies that will benefit the research. We will support uptake of the data and new tools by use of collaborative open access repositories, such as Open Science Framework and Protocols.io, to share experimental design, methods, materials and data with collaborators and other researchers. New datasets generated will also be made publicly available as a resource during and beyond the lifetime of the project using repositories such as MetaboLights and GEO. Posters and slides presented at scientific conferences, as well as unsuccessful approaches, will be made publicly accessible by publication in F1000Research. The license holder is a member of the various national and international grant committees, so will use these positions to promote the project and maximise the outputs.

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.

The project will use the mouse (including genetically modified mice) as a model system as the mouse is the only mammalian system available for modelling complex brain defects and testing their behavioural consequences. This includes a mouse model of the mitochondrial disease Leigh syndrome, which replicates many of the symptoms seen in patients. As we are studying brain development and degeneration, we will use both embryonic, juvenile and adult mice to understand how disease processes affect the brain at all stages. All the manipulations and tests we will use are established protocols that are optimised such that the fewest animals are used and distress or suffering minimised.

Typically, what will be done to an animal used in your project?

The majority of mice will spend most of their lives housed in appropriate conditions, in small colonies and will not experience any adverse effects. Mice will be bred to produce new animals to propagate the strains we require for our experiments. Cohorts of mice will be used for behavioral analysis that are non-harmful and may actually enrich the environment. Occasionally it will be necessary to perform injections on experimental cohorts to test new potential therapeutic agents. Mice that model early onset neurodegenerative disease will be closely monitored and humanely killed before their condition becomes severe. Some mice will be induced to have seizures as a model of mitochondrial disease.

Some mice will have surgical procedures to implant subcutaneous pellets or mini-pumps. Some mice will have surgical procedures to implant electrodes so that electroencephalography (EEG) or electromyography EMG) recordings can be performed. During EEG/EMG recordings mice will be single housed and tethered to a recording device but will be able to move freely.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of mice are not expected to exhibit any harmful phenotype. Due to the need to model a serious human neurodegenerative disease, a minority of mice will suffer from loss of body weight, hair loss and balance problems. Some mice will experience seizures for a maximum of a few minutes.
These will mice may experience anxiety and stress on induction of seizures via inhaled drugs or hyperthermia. Some mice may experience discomfort or pain associated with administration of substances, during or after surgical procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of protocols we will use are mild and we expect very few adverse effects (75% of mice). A minority of the animals (25%) will be used in a moderate protocol, but these animals will be carefully monitored to minimise suffering. At the end of the experiments the animals will be humanely killed and tissues may be investigated biochemically.

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?

Mammalian neurodevelopment is a highly orchestrated and complex process and so many aspects of neurodevelopment and neurodegeneration cannot be modelled in vitro. Mice have been developed as by far the most sophisticated mammalian model for gene manipulation and so are the most suitable model for investigating the function of the genes and pathways we are interested in. We intend to study the role of specific genes in neuronal development and neurodegeneration and so GM mice provide the best tool to achieve this goal. By using complete and conditional gene activation/inactivation models we can perform the required experiments using the fewest animals. Many of the neurodevelopmental and functional processes that occur in humans are conserved in mice that, combined with their sophisticated genetics, make them the ideal model for this project.

Which non-animal alternatives did you consider for use in this project?

We considered using invertebrate (Drosophila) models, cell culture models and iPSC models as alternatives for use in this project.

Why were they not suitable?

Much of the preceding work has been performed in Drosophila where we have identified new genes and molecular pathways that regulate neurogenesis and neurodegeneration. Where hypotheses can be tested in Drosophila we are and will continue to use this invertebrate model. We will use ex vivo cultures for some aspects of the work where appropriate, but these will not fully recapitulate the complexity and interconnected nature of the mammalian nervous system, which necessitates a live animal CNS model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The project will use the mouse (including genetically modified mice) as a model system as the mouse is the only mammalian system available for modelling complex brain defects and testing their behavioural consequences. All the manipulations and tests we will use are established protocols that are optimised such that the fewest animals are used (maximum of 3,300 over 5yrs) and distress or suffering minimised.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Wherever possible multiple readouts will be analysed on each animal, so the number of animals used are minimised. We will also use the minimum number of animals possible to obtain statistically significant results in our experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice will be bred to produce the minimum numbers of animals required for experiments and breeding pairs will be separated so that excess litters are not generated. When we use new techniques, or methods we have not used before, pilot studies will be performed on a small number of animals before starting the experiment in full. Tissue will be used for as many different experiments as possible to minimise the number of animals used and negate repetition of experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.
We will use genetically modified mice for the project. Mice have been developed as by far the most sophisticated mammalian model for gene manipulation and so are the most suitable model for investigating the function of the genes and pathways we are interested in. We intend to study the role of specific genes in neuronal development and neurodegeneration and so use of GM mice provide the best tool to achieve this goal.

Mice used in this project will be subjected to minimal stress and many of the protocols only require breeding and maintenance of colonies followed by killing to obtain tissues for analysis.

Why can’t you use animals that are less sentient?

We are using mice to model neurodevelopmental and neurodegenerative processes. The brain is the most complex organ in the body and using a species that are less sentient would not replicate the development and degeneration that occurs in the human brain. For

behavioral studies it is essential that we use live animals that are able to navigate behavioral paradigms if we are to understand how specific genes and pathways control neurological function.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use mouse models of mitochondrial disease that have a reduced threshold of induced epileptic seizures. We induce seizures primarily using hyperthermia. Pharmacological seizure induction will be used as a secondary method to validate phenotypes identified using hyperthermia induced seizures. Hyperthermia seizure induction is the more refined method as it does not expose animals to a pharmacological agent, which could have effects beyond the period of exposure. For hyperthermia seizure induction, temperature monitoring with a rectal probe is the most refined method as we need to measure core body temperature and so cannot use a probe inserted subcutaneously. Also, the rectal probe is temporary and does not require anaesthesia, which would be required to insert a telemetric temperature monitoring device. If a mouse exhibits a seizure, we will cull it if the episode reaches beyond a modified Racine scale 3 (reaches scale 4 or 5). Exposures will normally last 30 minutes but may extend up to 60 minutes if no seizure is observed. Mice are continuously monitored using CCTV.

EEG/EMG will be refined by only performing these procedures on a subset of mice to confirm electrophysiological activity typically associated with seizures.

Racine scale:

No change in behaviour

Sudden behavioral arrest, motionless staring (with orofacial automatism) 2 Head nodding Forelimb clonus with lordotic posture.

Forelimb clonus with rearing and falling.
Generalised tonic-clonic activity with loss of postural tone, often resulting in death, wild jumping.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the NC3Rs 'Responsibility in the use of animals in bioscience research', the Home Office 'Code of practice for the care and accommodation of animals' and use the NC3Rs 'Experimental Design Assistant' for practical guidance 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 receive regular updates from the NC3Rs and will implement any relevant advances into our project. We will also attend one of the regular AWERB meetings and attend a conference with a 3Rs focus, such as the Laboratory Animal Science Association (LASA) annual conference, or the World Congress on Alternatives and Animal Use in the Life Science.

Home Office

186. Respiratory Pharmacology II

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Asthma, COPD, Respiratory, Disease

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?

The development of therapeutic treatments for respiratory lung diseases. Initially working on the development of new vaccines against respiratory diseases. Further areas maybe added via amendment as additional programmes are developed.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Asthma UK calculated that the annual cost (direct and indirect costs) of treating asthma in the UK to be £1.1 billion, at least £666 million is spent on prescription costs each year. COPD costs the UK £1.9 billion each year in treatment. According to the UK government statistics in 2018, nearly 4 million work days were lost in the UK due to respiratory conditions.

Respiratory disease affects one in five people and is the third biggest cause of death in England. There is a massive unmet need for new treatments to cure or improve the management of these diseases worldwide.

The data generated during this project will be used to select the strongest candidates for potential new treatments. Likewise, new agents could be identified with the potential of increasing the range of medicines available in the fight against respiratory diseases.

What outputs do you think you will see at the end of this project?

Research undertaken on this project licence will result in efficacy, safety and tolerability data for potential new vaccines and antibody treatments against respiratory diseases and increase the understanding of the underlining biology behind the diseases investigated.

The data generated under this project licence will be used to progress the strongest candidates into clinical development with the intention of developing marketable vaccines. In addition data will help us understand the biology behind these diseases.

Data will also be used for filing new patents and thus disseminated through the patent publication pathways.

In addition, to patent applications, scientific publications and conference presentations will be used to disseminate key scientific findings and promote the general advancement of the research studied.

Who or what will benefit from these outputs, and how?

In the first instance the data generated from studies will aid the researchers in the selection and characterisation of new vaccines and antibody treatments which will lead to their further development (e.g. in clinical trials) and could potentially lead to new therapies for respiratory diseases being introduced to the market.

Long term, these products have the potential to significantly enhance the quality of life for people suffering these chronic diseases or potentially cure them. This will benefit the whole society via reduction in absenteeism from work or school and reduction in demand on health services.

How will you look to maximise the outputs of this work?

Our commercial client will, where not confidential, look to publish the information via scientific publications and conference presentations in addition to patent applications.

One of the key goals of our academic clients will be to publish the results via scientific publications conference presentations, in order to promote the general advancement of the fields studied.

Species and numbers of animals expected to be used

- Mice: 3600
- Rats: 3600
- Guinea pigs: 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.

Mice and rats are the lowest vertebrate groups on which well characterised and minimal severity respiratory disease models have been developed. Guinea pigs are widely used for respiratory research because their airways are generally more sensitive than other rodents, especially to allergens. Mice will be the preferred species used as an experimental model of airway disease. However, the final choice will be dependent on, the specific disease area, e.g. guinea pigs are a good species to use when investigating allergic bronchial asthma and anti-asthmatic drugs because the airway anatomy and the response to inflammatory mediators is very similar to humans. Also, further work planned e.g. specific disease models (to be undertaken by the client) would affect the species of choice.

In addition, these species, especially mouse, are the long-standing choice of immunologists with lots of historical data available for hypothesis-building and predictions and have successfully identified the key operating principles of the immune system on which major clinical advances are based, e.g. the successful development of PI3Kd inhibitor for respiratory diseases.

Typically, what will be done to an animal used in your project?

After arrival all animals will be allowed at least 7 days to become acclimatised to the unit.

After which the animals will be dosed with potential new vaccines or antibody treatments. These animals may receive up to total of 28 doses. A certain proportion of the animals will be used to investigate the effects and pharmacokinetic properties of the assigned treatment after single and multiple doses. Blood samples, and tissues taken post-mortem are analysed.

The next subset of animals will used to study the efficacy of the candidate vaccines or antibody treatment. The animals will be studied in a mechanistic model of respiratory inflammation, where they are challenged with the respiratory virus the treatment is targeted for, into the lung and the efficacy of the treatment on the inflammatory response investigated.

Another subset of animals will be studied in the ovalbumin or house dust mite induced respiratory disease models of either asthma or COPD. These animals will receive one of more doses of inflammatory agents and will develop a phenotype very similar to the human diseases. The ability of the candidate vaccines to alter this phenotype will be studied.

The last subset will also be studied in the same respiratory disease models, with an added viral challenge to study the ability of the candidate vaccines or antibody treatment to reduce viral exacerbations.

A proportion of the animals in each study will receive vehicle only these will be the control animals and are the group most likely to show the possible adverse effects. From experience we know the dose of challenge agent that gives the maximum response we

are measuring while keeping any adverse effects low if at all. This is especially important for these control animals.

While the maximum length a study could run for under this licence is 9 months, this would be very rare and the justification to run such a study would need to be strong. The vast majority of studies will be much short with a typically study lasting 2 to 3 weeks from starting the study to the final sampling time point. This would typically be a week of pre-dosing followed by a challenge and up to 2 weeks of sampling post-challenge.

What are the expected impacts and/or adverse effects for the animals during your project?

Studies conducted under this licence may induce some adverse effects in some of the animals. Typical adverse effects include changes in appearance, for example, minor changes in respiratory patterns ruffled fur, or changes in behaviour, e.g. the animals may become subdued. Other effects may include reduction in body weight and/or reduced eating. The larger proportion of animals used in these studies will, however, not experience any noticeable adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the majority of animals, the severity level will be mild. However, as stated above, in some studies the animals may experience some adverse effects, but these would only cause the animal a moderate level of distress which will in most cases be transient.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?
Understanding the mechanisms of action and assessing the effectiveness of potential new vaccines or antibody treatment in respiratory diseases requires the presence of a fully developed and functional respiratory system. Currently this cannot be reproduced outside of a living organism. Indeed, the assessment of the efficacy of candidates for respiratory diseases cannot be efficiently modelled in vitro due to the complexities of the lung microenvironment and the involvement of the immune system in these diseases. This cannot be fully replicated in a laboratory setting.

Which non-animal alternatives did you consider for use in this project?

Novel vaccines or antibody treatments selected for testing in the models contained within this licence will have to have been through a defined screening cascade of in vitro assays prior to testing in vivo models to show efficacy with in vitro readouts, such as, potency/selectivity, cellular function and ADME assays. This will minimise numbers of

candidates to test in vivo by implementing rigorous in vitro screening of candidates with high bars set for in vivo translation.

Why were they not suitable?

Improved in vitro screening techniques will reduce numbers of animals used by reducing the numbers of candidates that qualify for in vivo screening and will at the same time improve the quality of candidates brought forward for in vivo screening.

However, there is a point in biological research when in vitro experiments cannot provide all the necessary conditions to further research. In vitro models can mimic certain aspects of the of disease. They cannot, however, reproduce the complex interactions between different cells and mediators or reproduce the functional changes that occur as part of the ongoing disease process. The scientific community has established extensive in vitro systems to confirm the existence of a particular mechanism. But it is only with in vivo experiments that we can establish whether new medicines targeting such pathways will be efficacious and safe.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A typical experiment may include up to 60 animals and we might run approximately 60 studies, for rats and mice and 35 with guinea pigs over 5 years testing new medicine candidates. Thus, the expected number of animals to be used under this licence is estimated at 3600 mice, 3600 rats and 2100 guinea pigs.

Animal numbers are calculated using the lowest numbers per group to give reliable data based on previous experience. Number of studies per year are based on current rate of studies from the previous licence These numbers may be lower if alternative methodologies to replace such studies are developed over the next 5 yrs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure we use minimal number of animals required to obtain meaningful and relevant data, we have extensively consulted available literature, attended experimental design and statistical courses, discussed with statisticians and NC3R staff and information provided by the NC3R.

If the defined progression point for a candidate drug is a set blood plasma level 24 hour after administration. A study need only include a very early time point, to show the drug has successfully been administered and the decision-making 24 hour time-point. Additional time-points would almost certainly not influence the candidate's progression and therefore are not needed in the study. This significantly reduces the number of animals required. Also, it allows for more than one candidate to be tested in a study, reducing the

number of animals needed for control groups.

Wherever possible cassette dosing will be used in PK studies. This also reduces 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?

In many cases, the numbers of animals required will be reduced by longitudinal measurement of responses, e.g. by serial blood sampling or by optimised protocols to only include the key decision- making 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.

Mice rats and guinea pigs are the ideal organisms for these investigations:

They are the long-standing choice for respiratory research. There is an immense spectrum of well- characterised models developed over the years which cause minimal distress and suffering while providing meaningful data This is due to the similarities between the respiratory and immune systems in these animals and human. The commonly used laboratory strains of animals are well phenotypically and genetically characterised. Guinea pigs are widely used for respiratory research because their airways are generally more sensitive than other rodents, especially to allergens.

Secondly, because of the large number of well-characterised models, there are reagents available permitting the thorough, incisive, and comprehensive analysis of samples collected allowing for the maximum amount of information to be obtained from the experiments undertaken.

To minimise discomfort of repeated procedures such as anaesthesia, we will combine treatments under a single anaesthetic event wherever possible. The anaesthesia will preferably entail the use of inhalation agents whenever possible. Least invasive route of substance administration and needle gauge will be used where possible.

Negative control groups (baseline groups) will be minimised whenever statistically feasible.

Why can’t you use animals that are less sentient?

The human respiratory and immune systems are intricately complex and modelling it for assessment of new medicines requires models in vertebrate animals whose systems have been studied and can be, to a good degree, compared to human. Mice, rats and guinea pigs are the lowest vertebrate group on which plethora of reliable information on the

function of the systems are available and where well characterised and minimal severity respiratory models have been developed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise discomfort of repeated procedures such as anaesthesia, we will combine treatments under a single anaesthetic event wherever possible. The anaesthesia will preferably entail the use of inhalation agents whenever possible. Least invasive route of substance administration and needle gauge will be used where possible. Negative control groups (baseline groups) will be minimised whenever statistically feasible.

All animals will receive appropriate operative care in terms of anaesthesia and pain management both during and after the procedure.

In house expertise further enhances animal welfare, by providing close collaboration with dedicated animal care staff and veterinary consultants, and ready access to highly skilled advice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein. (Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs.)

Animals will continually be monitored for signs of pain and distress, especially post-challenge, by use of the grimace scale; https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/MGS%20Manual.pdf.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs are regularly published on our internal Users News Forum and other relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

187. Spine surgery: a new treatment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Orthopaedic, Surgery, Minimally invasive, Ultrasound, Implant

Animal types	Life stages
Cattle	adult
Sheep	adult
Deer	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 is to assess the safety and feasibility of novel medical devices, injectable implants, test articles or test substances used either as part of or in association with surgical procedures, or as part of or in association with non-invasive or minimally invasive procedures for tissue disruption or treatment, or for the treatment of conditions which may be simulated by surgical procedures. The context of this work is spinal disorders and in the first instance we are focusing on lower back 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?

Around 500 million people suffer from lower back pain, the 1st cause of disability and the 3rd largest cause of healthcare spending ($87.6 billion) globally. Patients with persistent,

recurrent lower back pain currently only have two diametrically opposed treatment options: conservative therapy, involving either physiotherapy or minimally invasive injections; and major surgery, most commonly spinal fusion, a complex high-cost procedure associated with significant complications ultimately leading to reduced range of motion and quality-of-life. In 2017/2018, the NHS undertook 211,000 pain injections and 52,523 spinal procedures, whilst in the US there were 9m treatments and 520,000 surgeries. Across Europe and the US, some 120,000 patients present with discogenic pain and an intact annulus, the initial target population for the technology at the heart of this application. The aim is to provide these patients with an intermediate treatment option, enabling percutaneous, minimally invasive nucleus pulposus replacement as a day case procedure, at a fraction of the cost ($15K vs $113K) of current surgical options, and leading to restoration of spinal function.

What outputs do you think you will see at the end of this project?

At the end of this project, in the first instance the expectation is a validation of the new spinal treatment and prototype, plus the collection of data for regulatory submission.

Another contribution would be a better understanding of the anaesthetic protocol for deer and a refinement of the anaesthetic protocol alongside the project. This output could become part of a publication.

Who or what will benefit from these outputs, and how?

The outcomes from this study will feed into a clinical trials application that has the potential to impact sufferers of lower back pain. Around 500 million people suffer from low back pain, the 1st cause of disability and the 3rd largest cause of healthcare spending ($87.6 billion) globally. Patients with persistent, recurrent lower back pain currently only have two diametrically opposed treatment options: conservative therapy, involving either physiotherapy or minimally invasive injections; and major surgery, most commonly spinal fusion, a complex high-cost procedure associated with significant complications ultimately leading to reduced range of motion and quality-of-life. In 2017/2018, the NHS undertook 211,000 pain injections and 52,523 spinal procedures, whilst in the US there were 9m treatments and 520,000 surgeries. Across Europe and the US, some 120,000 patients present with discogenic pain and an intact annulus, the initial target population for the technology at the heart of this application. The aim is to provide these patients with an intermediate treatment option, enabling percutaneous, minimally invasive nucleus pulposus replacement as a day case procedure, at a fraction of the cost ($15K vs $113K) of current surgical options, and leading to restoration of spinal function.

How will you look to maximise the outputs of this work?

The outputs from this work will be disseminated to the wider scientific community through publication in peer reviewed journals and by presentation at international meetings. Negative data will also be published and shared within the scientific community. Where appropriate, patients and the public will be informed of the outcomes through appropriate avenues.

Where appropriate, this work will lead to patent applications.

Species and numbers of animals expected to be used

- Cattle: 80
- Sheep: 80
- Deer: 80

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 non recovery studies approximately 20 animals in total will be used; this may be composed of deer, and / or sheep and / or cattle.

For recovery studies up to 60 animals in total will be used (determined to be the best species from the non recovery studies); this may be composed of deer, and / or sheep, or cattle.

Adult / juvenile animals are ideal for this study for fulfilling scientific objectives, based on anatomy, size and expertise of scientists and animal care staff.

Typically, what will be done to an animal used in your project?

Animals will be acclimatised and trained for handling purposes.

Each animal will be anaesthetised on up to 2 occasions and up to 5 intervertebral discs will be treated. Analgesia and antibiotic treatments will be provided post recovery and animals will be monitored by clinical observations.

The animals for the long term study will go through a recovery period of up to 12 months following the final procedure and up to 30 blood tests could be performed for the assessment of the long term effects.

Animals will be killed and post mortem assessments performed.

What are the expected impacts and/or adverse effects for the animals during your project?

Some short-term mild pain may be expected following the procedure, which will be controlled with appropriate treatment. Any adverse effects resulting from treatment failure will result in humane killing.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

For recovery studies, moderate in all animal types.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are needed to test this procedure and implant because there is no other alternative that models the acoustic and mechanical properties of a live spine while providing inflammatory, nerve, mechanical, anatomical and behaviour responses.

Which non-animal alternatives did you consider for use in this project?

We considered the use of human cadavers and computer modelisation.

Why were they not suitable?

Computer models were use to to refine the design of our prototype but failed to provide any inflammatory, nerve, mechanical, anatomical and behaviour responses.

Human cadavers were not suitable as gas is entrapped in the tissue and blood vessels after death and prevents any attempt to use ultrasound for both therapy and imaging. Embalmed human cadavers were not suitable as the mechanical properties of the tissues were changed having both an impact on non-external technology such as ultrasound and invasive techniques such as needle introduction.

Human cadavers failed to provide any inflammatory response, and any biological live feedback of the procedure.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The present project is divided into a non-recovery pilot study and a long-term recovery study.

Please note that sheep and cattle may not be used if our estimated best approach with the deer is conclusive. This will be answered by the initial pilot study, prior to recovery long term studies.

The pilot study is designed as well to spot at an early stage any obvious correction of the protocol that would be required, and the rough quantification of the observed effect can also be assessed.

The animal number for preclinical safety and efficacy studies will be based on the requirements of the relevant testing protocol for the regulatory authority to obtain the preclinical evidence on safety, biocompatibility and biomechanical.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Initially, two deer cadavers have been used to evaluate the suitability of the deer as a model and to optimize anesthetic protocols.

Then, a readjustment of the total number of animals will be made based on the non recovery experiments results and the observed effect.

Finally, using within the same animal up to 5 spinal segments for the treatment groups will reduce the number of animals needed by up to 5 times.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A previous pilot study on 5 sheep under a different license was used to refined the animal model for this study.

Then, two deer cadavers had been use to evaluate the suitability of the deer as a model and to optimize anesthetic protocols.

Computer modelling: The acquisition of x-ray imaging during or after the procedure will be used to model and adjust the experimental procedure and re-assess further animal need.

Cadavers will be shared with other groups where possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Female juvenile to adult deer and cattle will be used.

Deer and cattle have been selected as their intervertebral discs space and intervertebral disc type of cells are similar to human.

In the first instance, the surgical method involves minimally invasive techniques designed to minimise peri-operative pain and replace the disc structure with an implant capable of withstanding the appropriate mechanical strain. In initial cadaver and imaging studies already performed by the PPL holder, deer and cattle have a correct anatomical disc

space and furthermore, in recovery studies will provide an optimal model.

Optimisation will be performed under terminal anaesthesia. Once the protocol is defined and refined, recovery studies with general anaesthesia will be started.

Local anaesthetic may be used wherever possible, for example, to reduce stress prior to taking blood samples.

Why can’t you use animals that are less sentient?

The human spine is unique as human are bipedal and have a vertical load bearing. Only mammals can provide the relevant spine mechanical properties, inflammatory response. The prototype that we are creating is unique and is very dependent on the size and the geometry of the subject which requires large animal models. At a later stage, among the large mammals only a subset is losing notochordal cells at an adult age like human.

Terminal anaesthesia studies are being used for the pilot study to refine protocols.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise discomfort of repeated procedures such as anaesthesia, we are combining treatments where possible under a single anaesthetic event. The anaesthesia will preferably entail the use of inhalation agents whenever possible. Least invasive route of substance administration and needle gauge will be used where possible. Negative controls are within the same animal.

All animals will receive appropriate operative care in terms of anaesthesia and pain management both during and after the procedure.

Expertise at facility further enhances animal welfare, by providing close collaboration with dedicated animal care staff and veterinary consultants, including animal care staff and a veterinarian with previous experience of working with deer, as well as other large animal species, and ready access to further highly skilled advice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein. (Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs.)

Animals will continually be monitored for signs of pain and distress, especially post-challenge, by experienced veterinarians and animal care technicians with significant experience in these species.

Anaesthetists work to best practice guidelines for large animal anaesthesia and maintain CPD to keep up to date with new practices.

Standard Operating Procedures are employed for animal preparation, surgery and

recovery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs are regularly published on internal News Forum and other relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

188. Testing and development of vaccines/therapeutics for influenza

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

influenza, vaccines, antivirals, transmission

Animal types	Life stages
Ferrets	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 intend to assess new vaccines and therapies which are required to prevent and treat disease caused by influenza. We need to know if these have biological activity before we try to use them in people.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project will allow for the use of established animal models and their further development. This will inform studies into viral pathogenesis, transmission and efficacy of vaccines and medicines that have most clinical relevance.

The data collected will enable the selection of potential vaccines and medicines. This will be of benefit to humans because flu outbreaks can harm or even kill large numbers of people.

We will also improve the data available to scientists about how ferrets respond to flu and the vaccines and medicines that treat it. This will benefit humans because in the future, vaccines and/or medicines will be selected faster and with a greater likelihood of success.

What outputs do you think you will see at the end of this project?

Outputs will include:

- Refinement of the ferret model of influenza; new information on the model which will lead to publications and dissemination of information to the wider influenza and animal modelling field.
- Pre-clinical evaluation of novel vaccines and medicines against influenza.
- Contribution to the successful release of the live attenuated influenza vaccine for use in school age children in the UK.

Who or what will benefit from these outputs, and how?

This licence application will contribute to the understanding and refinement of the ferret model of influenza allowing long term contribution to the field.

This licence application will also enable us to select good candidate vaccines and medicines from a range of candidates. By filtering these candidates through our models of infection, we will reduce the number of candidates required to be tested in humans and advance translational research.

Additionally this licence will allow a key component of the live attenuated influenza vaccine testing to be completed. This will contribute to the continued rollout of the vaccine in school aged children in the UK which is part of the vaccination schedule as recommended by the Joint Committee on Vaccination and Immunisation.

How will you look to maximise the outputs of this work?

In addition to peer-reviewed publications, the work performed under this licence will be disseminated widely at international conferences and the data provided to customers to facilitated the generation of products which will have direct benefits to human health.

Species and numbers of animals expected to be used

- Ferrets: 2600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

Ferrets are the gold-standard animal model for influenza. Both juvenile and adult ferrets will be used during this project.

Juvenile ferrets will be used in the release testing of an influenza vaccine.This test is regulated and forms part of the vaccine release testing.It is authorised by regulatory authorities such as the FDA and MHRA.

Adult ferrets are used for the challenge and transmission studies, as well as studies for the pre-clinical evaluation of novel vaccines and medicines.

Typically, what will be done to an animal used in your project?

Typically the following will occur during an experiment:

Following acclimatisation animals will be sedated and undergo a baseline bleed to ensure that there has been no prior exposure to influenza.

Animals may then be vaccinated, most commonly intramuscularly or intranasally. Alternatively therapeutics will be administered, most commonly intranasally or orally.

If a vaccine is being assessed, the duration of the experiment can range from one week to several months depending on the length of a vaccination phase.

Clinically observations are made prior to administration of influenza.

Influenza virus will then be administered to ferrets, usually via the intranasal or aerosol routes.

Once influenza has been administered the experiment is usually completed 14 days later. Clinical observations are performed at least, daily.

Animals can be expected to be sedated and nasal washed/swabbed daily (up to 8 times sequentially) in order to study the shedding of the virus. Animals can be sedated and bled approximately 5 times over a 14 day period (ensuring only a maximum of 10% of total blood volume is taken at one time and no more than 15% is taken over a 28 day period).

At the end of all studies, animals will be euthanised by a Schedule 1 method or by terminal exsanguination under full anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential adverse effects to virus challenge, anaesthetics and administration of substances have been identified.

Expected effects of ferrets having the influenza virus are sneezing, nasal discharge, lethargy and loss of appetite. They may lose weight and they may have a temperature.

Adverse effects associated with the viral challenge will be identified by detailed behavioural monitoring. Together with this behavioural monitoring, objective data (animal weight and temperature) will be collected and analysed.

Ferrets that have been anaesthetised may become dehydrated and could develop hypothermia, but this is unlikely due to refinements put into place.

Any adverse effects associated with the anaesthesia will be monitored and appropriate steps taken to minimise hypothermia and dehydration.

Ferrets may experience mild discomfort when being handled during the administration of influenza virus, vaccines or medicines.

Adverse effects associated with the administration of sub-stances will be minimised by using highly trained staff competent in the delivery of substance via various routes, by using the smallest of volumes and rate of administration of substances.

At the end of all studies, animals will be euthanised by a Schedule 1 method or by terminal exsanguination under full 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 from influenza infection in ferrets is expected to be moderate at most. Depending on the strain of influenza used animals can be expected to experience a mild to moderate severity.
Clinical symptoms are expected to resolve after 3 - 6 of onset.

Humane clinical endpoints have been clearly defined therefore, unnecessary suffering is avoided. At the end of all studies, animals will be euthanised by a Schedule 1 method or by terminal exsanguination under full anaesthesia.

What will happen to animals at the end of this project?

- Killed

Replacement

**State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?**

The ways in which the human airways protect themselves against influenza viruses and the ways in which the immune system works is complex and has not yet been successfully modelled in computers. We cannot show whether a vaccine or medicine can both reduce symptoms and prevent progression of disease in the body except by using animals

Which non-animal alternatives did you consider for use in this project?

Limited trials can be conducted in human volunteers. There are no suitable non-protected animal alternatives in which to model human influenza.

Why were they not suitable?

There are ethical and safety reasons that prevent us from studying what we aim to study in this licence in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of animals used during the five-year period of this licence is based on the numbers of ferrets used on the previous version of this licence and refinements made during its use.

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 per group will be used to generate data that is useful. Statistical advice is available and this advice will be used to minimise animal usage in studies.

Regulatory testing of the influenza vaccine involves relatively small numbers of animals per test and multiple test samples (same strain) can be run using the one control group which will subsequently reduce the overall number ofanimals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Studies will be conducted in a step-wise manner so that the number of animals used will be minimised if the treatment shows no likelihood of working; for example, if a new vaccine does not elicit an appropriate immune response, then it would not progress to a challenge efficacy study.
Robust scientific quality control of the test materials and methods will ensure that studies are carried out successfully first time, minimising the need to repeat studies and subsequently reduce the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The work carried out under this licence will involve the use of ferrets as they possess numerous advantages over other animal models, making them the most suitable animal model for the range of studies proposed within this licence.

To assist in the prompt recognition and subsequent intervention, critical periods will have been identified and monitoring frequency increased to every six hours as a minimum. Humane clinical endpoints have been clearly defined, therefore, unnecessary suffering is avoided.

In order to provide animals with maximum social interaction and environmental enrichment we will aim to group house, where possible, animals within the containment facility throughout the duration of the study.

Regulatory testing of the influenza vaccine involves relatively small numbers of animals per test and multiple test samples (same strain) can be run using the one control group which will subsequently reduce the over-all number of animals used.

Why can’t you use animals that are less sentient?

As in humans, influenza illness in ferrets is acute and usually lasts 3 – 5 days. While several other animal models have been utilised to study influenza, unlike ferrets the disease progression does not resemble that in humans (nasal discharge, sneezing). Aspects of this licence rely on the ability to observe clinical signs in the infected animal, for example antiviral testing upon symptom onset.

Regulatory testing of the influenza vaccine requires the use of juvenile ferrets as licenced by regulatory authorities.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The humane endpoint for all studies is a combination of detailed behavioural monitoring (nasal discharge, sneezing, lethargy and loss of appetite and objective data collection (animal weight and temperature). As this programme of work progresses, it is intended that data collected will inform the refinement of subsequent studies i.e. Periodic review of humane endpoint 20% weight loss criteria to determine ifthis can be further refined.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For studies where regular blood samples are required standard guidelines for delivery and removal of substances via various routes will be followed as outlined in 'First report of the BVA/ FRAME/ RSPCA/ UFAW joint working group on refinement '(Lab Animals (2007) 41, 321-328) and Wolfensohn and Lloyd (2013).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed regarding advances in the 3Rs but keeping up to date with advances in the field on influenza and animal modelling. This will be done by attendance at meetings, conferences and discussion with our peers.

Discussion around advances in the 3Rs that will benefit the animals used under this project licence will be encouraged. These changes will be represented internal to the AWERB committee. Any changes that will be beneficial to the animals while maintaining the scientific integrity of the protocols will be implemented. Any changes will be made to the licence, if required, to implement advances in the 3Rs effectively.

189. Regulation of metabolism and body weight by the brain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Brain, Metabolism, Body weight, Feeding

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the brain controls food intake and body weight and, in turn, how changes in food intake and body weight influence the function of the brain and other organs in 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?

Having enough energy (in the form of food) is essential for survival of all organisms. We know that the brain is important is controlling food intake and body weight in animals, but we do not yet fully understand the underlying processes. It is important that we learn more about these on a molecular, cellular, and whole animal level so we can develop new treatments for diseases where these processes do not work properly. These including obesity (where there is too much food intake) and anorexia/cachexia (where there is too

little food intake).

What outputs do you think you will see at the end of this project?

The main outputs from this work will be new knowledge on how the brain controls food intake and body weight which will be shared publicly with the scientific and wider community via research publications, presentations at scientific conferences and other Universities, and also shared online via social media and our lab website.

Who or what will benefit from these outputs, and how?

The proposed studies address fundamental biological questions important for human and animal health. In the short-term the main benefits will be the contribution of new scientific knowledge. In the longer term, this knowledge has potential to provide information that informs those seeking novel treatments for obesity, diabetes, and weight-loss associated with disease.

Academic benefits (short-long term): The most immediate benefit of this work will be to other scientists. After publication, the findings of our studies will be of interest to scientists, veterinarians, and clinicians in the UK and internationally working directly on the regulation of food intake, metabolism, and body weight. Furthermore, as reduced food intake and body weight often occurs with other diseases, including cancer and dementia (where it contributes directly to worsening of health), the data may be of significance to a wider audience studying these conditions. This includes those working in the pharmaceutical industry to identify new treatments for disorders such as obesity, diabetes, and weight-loss associated with disease. In addition to addressing basic biological questions relevant to human and animal health, our work may be of relevance to those in the agricultural/veterinary sectors focused on manipulating feeding for food production or husbandry purposes.

Medical and veterinary benefits (long-term): With respect to human medicine, obesity currently directly costs the NHS £4.2billion annually due to the associated effects on health. There is a great need for improved cost-effective therapies to reduce this economic and medical burden. Surgical treatments are effective but invasive and costly, and there are limited qualified surgeons available. Unfortunately, currently available drug therapies for obesity are of limited effectiveness. As such, studies like the ones proposed are critical, as we need to improve our understanding of the mechanisms by which body weight is regulated by the brain. Similarly, the proposed research may also benefit veterinary and agriculture sectors by providing new mechanisms to treat obesity in companion animals and enhance weight gain in food production animals.

To maximise the benefit of our work, we will disseminate our findings promptly by depositing our research papers on open-access pre-print servers and publishing open-access in peer-reviewed scientific journals as soon as possible thereafter. To ensure that the research is communicated to the widest possible audience, in we will attend both specialty scientific conferences and broader meetings intended for a wider scientific and clinical audience. To maximise our opportunity for communication with industrial stakeholders, we seek to identify industrial partners who may be interested in our work and seek to communicate directly with them.

How will you look to maximise the outputs of this work?

To communicate our findings, we will: 1) publish our work (open access whenever possible) in the form of research papers; 2) post our research papers to the open-access

pre-print servers; and 3) present our findings promptly at national and international scientific conferences. To ensure that the research is communicated to the widest possible audience, in addition to attending specialty scientific conferences, we will also attend broader conferences intended for a wider scientific and clinical audience.

We will use online resources to share our research more broadly. We actively use Twitter to disseminate knowledge and we have a dedicated lab website including lay summaries of our work and publications. Our city, and the surrounding area, has a vibrant science communication scene with numerous annual public outreach forums. Members of our research team have delivered several presentations to the public at these local events. During the project, the applicant commits to continuing to engage in these public outreach activities.

Species and numbers of animals expected to be used

- Mice: 3,100

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.

This project will use mice. The have been selected as they are mammals which have similar hormone and digestive systems to humans, and their use to study energy balance has been extensively validated by the scientific community. They are readily genetically modified, including several strains which are commercially available.

Most studies will be performed in young or adult animals as these are free feeding and their behaviour can be readily monitored.

For some studies we will use mice which are a few days old as their cells grow readily in culture.

Typically, what will be done to an animal used in your project?

Typically, an animal will have its cellular activity or energy balance altered by diet, genetic modification and/or treatment with a drug. We will then measure how these manipulations impact the animals with respect to their food intake, body weight and blood glucose. Some mice will be killed so we can see how genetic modification and/or treatment with drugs changes their brain and other tissues on a molecular level. Some very young mice will be killed and their brain cells cultures for laboratory tests of cell function on a molecular level.

A typical experiment will last 2-8 weeks, including the time for the animals to get used to being handled and resting in between procedures.

Examples of typical series of experiments is as follows:

Example 1:

An animal undergoes surgery to have a virus that causes a genetic modification injected

into its brain.

Following recovery, the mouse is individually housed and then receives an injection of a drug which alters activity of its cells and has its activity and food intake non-invasively monitored in its home cage.

The following week the mouse receives another injection of the drug and a test is performed to see how this changes the way that their body copes with changes in blood glucose.

Following rest, the mouse is injected with the drug again and then killed and their brain tissue examined for molecular changes.

Example 2:

An animal is fed a high-fat diet for 4 weeks and its change in body weight monitored.

A test is performed to see whether the diet and weight change has altered the way that their body can cope with changes in blood glucose before and after the mouse is fed the high-fat diet.

Following rest, the mouse is then killed, and their brain and other tissues examined for molecular changes.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals on this project used for breeding or culture of cells are unlikely to experience any adverse effects beyond those that are mild or transient.

Animals which undergo surgery may experience some weight loss and pain following surgery, but they will receive post-operative care, be carefully monitored until recovered, and given pain relief medication. Typically, animals are fully recovered, including regaining any weight lost, by 7-days after surgery.

Some studies occasionally require the animals to have short periods without food (up to 16 hours). This may be done to: 1) examine how food deprivation causes molecular changes in the brain; 2) examine the way that genetic alterations or treatments with drugs change the physiology and behaviour of animals during food deprivation and/or after they get access to food again; 3) to control access to food so we can motivate animals to eat; 4) to reduce differences in blood sugar levels between animals before studying their ability to control their blood sugar levels. These will help us to understand the processes by which the brain recognises and responds to changes in food availability/energy levels.

Any periods without food will be as short as possible, and each animal will only experience this twice in an experiment. Animals that lose weight following food deprivation, typically recover rapidly, usually regaining any weight lost within 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)?

Based on our experience conducting similar experiments of this type in mice over the last five-years the expected proportion of animals in each category will be as follows:

Mild = 70%

Moderate = 30%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The regulation of food intake and body weight is a complex process which involves the communication of information between the brain and lots of other organs in the body including the stomach, intestines, pancreas, liver, muscle, and fat tissue. Each of these organs is also made up of a complex mixture of different types of cells which act cooperatively so need to be studied together in the context of the intact living animal.

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
- The use of non-protected species
- Human tissue samples

Why were they not suitable?

Computer modelling - Due to the complex systemic nature of energy balance and obesity it is impossible to model with a computer due to the infinite number of variables both known and unknown.

Cell lines - Cell lines will be used to validate the molecular activity/specificity of any drugs or therapeutic agents before they are used in animals, reducing the number of animals used. Analysis of cells in culture (generated from young mouse pups) may also be used where possible to allow further investigation of signalling pathways in the laboratory and minimizing the use of living animals.

The use of early developmental stages of protected animals - As the focus of this work is

on the effects of energy balance and obesity in adult animals, earlier stages of development cannot be used as they need to be free feeding and independent from their parents.

The use of non-protected species – In order to model the physiology of human metabolic disease it necessary to use an animal model with a comparable hormonal and digestive system and unfortunately non-protected species do not fall into this category.

Use of human tissue – The human tissue samples are collected post-mortem so cannot be experimentally manipulated to address the scientific questions that we have.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 this number based on our extensive prior experience with similar studies. This does not reflect the total number of animals which will receive experimental procedures which is likely to be a lot less. This number also includes the genetically modified animals which will be used only for breeding and the very young mice that will only be used for tissue to culture cells.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- In the group we actively use the NC3Rs experimental design assistant tool to design our animal studies. https://eda.nc3rs.org.uk/
- To promote reproducibility of our work by others, we also strive to follow the ARRIVE guidelines for experimental reporting of animal studies for all our published work - https://arriveguidelines.org/

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, the results of which are then used to mathematically calculate the minimum number of animals needed in the full study.

Where possible we group studies together so information from each animal is used optimally to address as many scientific questions as possible, this includes taking as many tissues from the animals as possible after they are killed and sharing them with others.

When breeding mice, we always optimise our methods to produce the minimum number needed for scientific and husbandry 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.

This project will use mice at different life stages.

Mice are the most used research model for the study of obesity and the regulation of energy balance; therefore, most of the existing research comes from studies in this model. As such, the mouse as a model to study obesity and the regulation of energy balance has already been extensively validated by the scientific community. Mice have a similar digestive system and hormonal regulation to humans. The mouse strain that we will use for this project gains weight and becomes obese readily when given a high-calorie diet for several weeks.

Mice can be genetically modified. Lots of different mouse strains with genetic modifications which will be used for this project have already been created. These will be used for “genetic dissection” of critical pathways mediating the regulation of food intake and body weight by the brain. Several genetic mouse models of obesity are already commercially available.

To date numerous genetic alterations first identified to cause obesity in mice have also been found to result in obesity in humans, validating the utility of the mouse as a model for human obesity which is caused by changes in genetics. A good example of this is the melanocortin-4 receptor. First implicated in mediating food intake and obesity through mouse studies, alterations in the melanocortin-4 receptor gene are believed to be responsible for up to 5% of cases of severe early onset genetic obesity in children.

We will always endeavour to use the least invasive approach possible to answer our scientific questions. We have an automatic system for remote continuous monitoring of feeding/drinking and activity in animals in a home-cage environment which enables the maximum information to be obtained with minimum handling of the mice. Where animals do need to be handled, we use non-aversive handling approaches where possible.

Why can’t you use animals that are less sentient?

As the focus of this work is on the regulation of food intake and body weight in adult animals, earlier stages of development cannot be used as they need to be feeding independently.

To model the physiology of human metabolic disease it is necessary to use a vertebrate model with a comparable hormonal and digestive system and, unfortunately, less sentient species than mice do not fall into this category.

Where possible we will use mice that have been terminally anaesthetised, but this is not possible when studying behaviours such as feeding where the animals need to be awake

and moving around. General anaesthetics also can change some of the measurements that we will be taking, such as blood glucose, so would confound the results.

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 minimizing adverse effects without impacting scientific outcomes.

Measures already employed to minimising suffering:

Housing and husbandry: For most studies, the animals will be housed in groups to reduce stress. For the studies where an accurate measurements of food intake and/or other behaviours is required, the animals will need to be individually housed. This is needed because when housed in groups, social hierarchy can cause unpredictable variations in food intake and behaviour which may increase the number of animals that need to be studied. To minimise stress, all mice (group and individually housed) will be provided with enrichment in their cage such as bedding, shelters, and chew toys. Animals will only be individually housed if absolutely necessary for scientific, welfare or husbandry purposes. Any individual housing will be used for the minimum time possible to meet the scientific aims of the study.

Behavioural studies: Where possible, automated systems for monitoring food intake and/or activity in a home cage environment will be used to minimise the need to disturb the animals. Where animals do need to be handled for experimental purposes, stress to the animals will be reduced by habituating the animals to the procedures, e.g., picking them up to be injected or weighed, and non-aversive handling used wherever possible. Body weight and food intake measures will be used during the habituation period as indicators of stress and the habituation period will be maintained until the animals' body weight stabilises and/or reaches at least their initial/pre-habituation body weight.

Breeding: We will not continue to breed any genetically modified mice that show negative/harmful symptoms.

When generating any novel mouse model with a genetic modification there is always a risk of unexpected effects. Most animals produced under this licence are not expected to exhibit any harmful symptoms. If they do, they will be closely monitored and may be humanely killed, if there is evidence of suffering that is greater than minor and transient or in any way compromises its normal behaviour.

Route of drug administration: In studies where administration of drugs or other therapeutic agents is required, the least invasive route of administration will be used to answer the experimental question to be addressed. Where possible oral (via food or water) or administration under the skin (subcutaneous) will be favoured.

Minimising Surgery: All surgical procedures will be performed using sterile aseptic technique to minimize the risk of infection. Pain relief will be given to all animals undergoing surgery to minimize suffering associated with post-surgical pain. Animals that undergo surgery will receive post-operative care and will be monitored regularly until recovered so that any signs of pain and or distress are identified as early as possible, and alleviated.

Duration of fasting: Fasting will only be utilised during our studies if absolutely necessary, e.g., to induce hunger and/or reduce differences in blood sugar levels between animals. To induce hunger, as mice eat approximately 70% of their daily food intake during the night when they are most active, typically food is withheld during the night period and reintroduced the following morning after the lights come on again. As mice are nocturnal and largely sleep during the day, fasting during the day period would have little effect on hunger. We may also withhold food/fast mice for shorter periods, typically 4 hours, to minimize feeding which might cause unpredictable variations in some experimental measurements, such as blood glucose.

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 (https://www.nc3rs.org.uk/experimental- design-assistant-eda) to plan our studies, which helps us to incorporate best practice for the 3Rs into our study design.

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/

LASA guidelines will be consulted for guidance on administration of substances.

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 us to incorporate best practice for the 3Rs into our study design.

We will effectively implement any advances in the 3R in our studies after consulting with the Named Veterinary Surgeons and Named Animal Care and Welfare Officer.

190. Investigation of normal haematopoietic stem cell subversion and the evolution, maintenance and targeting 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

Key words

Normal blood stem cells, Blood cancers, Evolution and maintenance, Therapy, Resistance

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 will determine what goes wrong with normal blood stem cells in processes such as cell growth and survival and how abnormalities of these lead to the development and maintenance of blood cancers.
We will then use this information 1) to help predict those likely to develop blood cancers, 2) to potentially intervene earlier in the course of blood cancer development and ideally prevent blood cancers from developing in those at high-risk and 3) to better treat those patients with established blood 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?

Blood cancers are devastating diseases that are associated with significant illness, with

many being fatal. They represent around 1 in 10 of all cancers and may be preceded by early/pre-cancerous conditions. Blood cancers are relatively common when considered as a group, occurring at a frequency of approximately 95 cases per 100,000 of the population (Surveillance, Epidemiology and End Result (SEER) programme data) when the pre-cancerous conditions also included. Established blood cancers are often incurable and caused 12,961 deaths annually in the UK in 2015-2017 (CRUK statistics).

Although they do affect the young, being the most common form of childhood cancer, blood cancers are predominantly diseases of the elderly and their incidence rises steadily with age. The age of the general population is rising steadily, and the prediction is blood cancers will become significantly more common over the next few decades. Moreover, some disease such as Non-Hodgkin's Lymphoma (NHL), the single most common blood cancer and the 6th most common cancer of all types, has been seen to be occurring more frequently than would simply be expected from the ageing of the population. Blood cancers are also not only associated with significant illness and death; although poorly effective in curing blood cancers, the treatments that we currently use are also highly toxic and extremely expensive (costing up to around £250,000 per patient if a bone marrow transplant, is used as part of the treatment). Thus, the treatment of blood cancers is an unmet medical need and costs the NHS more than £100 million annually.

This project is important as it aims to improve on the range and effectiveness of therapies for the treatment of blood cancers. It will do so by identifying new treatments for blood cancers, determining how resistance to existing and novel therapies develop and can be prevented and, through the study of early stages of blood cancers, will test the effect of and suggest the likely benefit of earlier therapy.
Multiple solid organ cancers are more easily treated and sometimes cured by treatment earlier in the disease course and we hope that this general observation will extend to blood cancers. It is also possible that we may be able to, in effect, prevent certain blood cancers from occurring. Work over the last decade has demonstrated that the mutations that have been shown to cooperate to generate clinically obvious blood cancers can sometimes be detected earlier in apparently “normal” individuals with no apparent blood disorder. This condition is called age-related clonal haematopoiesis (ARCH) or clonal haematopoiesis of indeterminate potential (CHIP), and will hereafter be simply referred to as clonal haematopoiesis (CH). Thankfully not all individuals who carry these mutations go on to develop a blood cancer, but the likelihood of this occurring is increased significantly in comparison to the general public and to those who do not carry these mutations. Although our understanding of how individual mutations contribute to the maintenance of established blood cancers is improving, with our own groups contributing significantly to that understanding, exactly how they change the function of the normal blood stem and progenitor cells that generate all the different blood cell types is poorly known. Similarly, the progressive events that lead to the development of newly diagnosed and treatment-resistant blood cancers are very poorly understood, and this lack of knowledge hampers our attempts to improve treatment outcomes.

Blood cancers originate in haematopoietic (blood) stem cells (HSCs). These cells have the ability to remake themselves by dividing to generate daughter cells with exactly the same properties; a process known as “self-renewal”. This ability may be an in-built feature of the cell (such as in a normal HSC) or it may be acquired, i.e. due to the effects of a mutation occurring in that cell. Like all cells within the body these stem cells are constantly exposed to external stresses and influences that can lead to the development of mutations. These mutations alter the code of DNA and through this the information contained within DNA to control the behaviour of the cell. Thankfully, the vast majority of these mutations are fully

repaired, however small numbers of mutated cells can persist. Unlike the majority of other cell types that simply mature and eventually die, HSC possess self-renewal and can therefore retain the mutations within their DNA by passing them onto their daughter cells. Whilst the vast majority of such mutations are “neutral” and do not affect the behaviour of the HSC, certain mutations can equip an HSC with an advantage over time, allowing its daughter cells to expand, which gives rise to clonal haematopoiesis (CH). CH is driven by a group of mutations that are associated with the development of blood cancers, although this progression is not inevitable. Importantly, CH becomes increasingly common with age and is detected in more than 30% of people aged 70 years and older. Similarly, specific long-lived stem-like progenitor cells within the immune system that carry immunological memory are also thought to develop mutations and predispose individuals to the development of the blood cancers derived from these tissues, lymphoma and myeloma.

In addition to mutations changing the internal behaviour of HSCs, it is clear that other factors externally influence the behaviour of HSCs during normal blood cell development and during the development of blood cancers. There is now increasing evidence that the rate at which an HSC and its daughter cells grow, is affected by ageing and external “stress” factors such as infection, inflammation and diet. HSC and progenitors reside in a specialised environment within the cavities of bones. This environment is called the bone marrow and the non-blood forming cells that HSC interact with are known as the bone marrow microenvironment (BMME). The BMME provides a protective and nurturing environment for the continued growth of HSC. It is also thought likely to produce the final signals of the above “stress” factors that directly stimulate the HSC and, in part, facilitate the development of blood cancers.
Moreover, it is thought that the BMME also provides survival signals to blood cancers once they develop and is a critical factor in the development of treatment resistance in blood cancers. However, how these signals change from the support of normal HSC to the support of blood cancer stem cells is unknown.

This project will investigate internal (intrinsic) and external (extrinsic) factors that allow the normal function and growth of HSC and how these factors are altered to initiate and maintain blood cancers, with the aim of identifying therapeutic targets to intervene to improve outcomes or potentially even prevent the formation of blood cancers.

What outputs do you think you will see at the end of this project?

The new outputs that we expect to see from this project will relate to new knowledge obtained about normal blood stem cell function and how this is altered to generate blood cancers. This information will also include identifying specific critical factors that control this process that might be useful as therapeutic targets, to treat or potentially prevent the development of these cancers, or as markers to diagnose them earlier in their development. As our project will involve therapeutic targeting of these candidates, we also aim to identify potential novel treatments that act on the targets and to generate enough data on their effectiveness to enable the treatments to be taken forward into clinical trials.

Our project will also generate new strains of genetically altered animals to be used as models for studying blood cancers which will be of great potential advantage to other researchers studying the same disorders.

We will publish our results and data with free access in reputable scientific journals and talk about it at major conferences. We will also deposit our data in standard archives so it is available to other researchers and can help to guide their studies. Our research will also

be of wide public interest and important to a number of major charities and patient groups and we will communicate our findings as broadly as we can, through press releases and by contributing to events organised by those groups.

Who or what will benefit from these outputs, and how?

In the short-term, the medical and scientific communities, as well as drug manufacturers, will benefit from our research by using our findings to help develop new strategies to identify, potentially prevent and treat blood cancers.

To facilitate the rapid benefit of patients, we plan to work along with other scientists, medical doctors and biomedical companies to speed up the application of our findings into new testing methods and new treatments. We have a great deal of experience in such processes and we are already advanced with trials of drugs discovered through similar studies in previous licences.

In the longer term, we hope that our work will lead to the development of new methods for the prevention and treatment of blood cancers. Prolonged survival and a better life quality for patients with blood cancers and their families will be the most direct and measurable benefits from our research. In the broad sense, ensuring better treatment options for patients will have a positive effect on society at many levels.

Our groups have a longstanding interest in public engagement activities and we work in or are associated with collectives and in institutions that have dedicated public engagement teams. We will remain actively involved in these activities to promote the understanding of our research, in line with the programme of public engagement proposed by our institutions.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. The generated data will be stored in an archive and made freely available to other researchers around the world via databases such as the European Nucleotide Archive (ENA) at the European Molecular Biology Laboratory- European Bioinformatic Institute (EMBL-EBI). Our new animal models, as well as tissues collected from the mice will be valuable to other scientists studying the development of blood cancers. We will distribute our mice and other tools freely to non-commercial establishments after publication. Pre-publication access will be extended to other scientists as part of collaborative studies. We have already exported a number of our mouse models to several leading universities and institutes worldwide. We will also aim to share and use tissues from aged mice using ShARM (Sharing Ageing Research Models, https://www.sharmuk.org).

Species and numbers of animals expected to be used

- Mice: 36,900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are widely used for biomedical research due to their anatomical, physiological and genetic similarity to humans. Furthermore, they are easy to breed and keep in a laboratory environment. The enormous progress of technology over recent years has enabled us to efficiently generate mouse models useful to study disease states such as the blood cancers that are our main research interest. Our mouse models carry changes in their genetic material (mutations) copying or resembling the changes in genetic material of patients with blood cancers.

The incidence of blood cancers increases with patient age. Consequently, the effects generated by the ageing on the blood system are an essential part of our project. In many aspects, the ageing of mice resembles the ageing of humans and therefore, mice are an extremely useful resource to understand human ageing. The life-span of a mouse averages around 2 years which allows us to complete the project goals within a reasonable time-frame. Equally, we will use young adult mice as a comparator and reference for results obtained with aged mice.

Our work is focused on the blood system and will benefit from a well-established specific range of methods that are already established and accepted for the mouse. For example, blood cells can be easily analysed and separated by flow cytometry, a method where various molecules on the surface of the cell are specifically tagged and caused to emit light under certain conditions. This labelling can be subsequently used to identify and separate out the cell for further analysis.

Typically, what will be done to an animal used in your project?

Most methods and procedures required for this project are well established in the field of blood cancer research and in our group. We have successfully used them in the past to characterise different mouse models of leukaemia and lymphoma.
In practice, mice will be bred to generate offspring that have alterations (mutations) of relevance in their genetic material (DNA), so-called genetic alterations. For the wellbeing of the animal, we normally engineer these alterations under our control, so that a mouse will need a specific “switch” involving a single or repeated injection of a substance that will turn the genetic alteration on or off. Some of these experimental mice, after activation of genetic alteration, may be kept alive until up to 2 years of age to assess the role of the ageing process, an independent risk factor in blood cancer formation. Throughout this time, we will collect blood from mice on one or multiple occasions to assess how the number of blood cells change, as one of the markers to assess disease progression in response to various treatments, either to potentially stimulate the development of blood cancers or as potential treatments for these cancers. Frequently, these treatments will aim to mimic the exposures normally encountered by humans during their lifetime, such as inflammation following infection or DNA damage. The treatments may last up to 16 weeks and are expected to produce long-lasting, but generally immediately undetectable changes, in the same way that occurs in humans. To this end, mice may be treated with different substances to modify either the behaviour of the abnormal blood cells or their normal body environment, the bone marrow microenvironment (the surrounding cells that support the growth of blood cells within the bone marrow). The treatment may involve a single or multiple administrations of a substance by different delivery routes (e.g. in the food, injected under the skin or into the body). The frequency of these treatments and volumes used are not expected to harm the animals. Examples of cell-targeting substances include drugs that have been shown to effectively inhibit tumour cell growth

when tested "in a test-tube". Some mice will undergo treatment with radiation, either as a challenge to the haematopoietic system, and/or to remove their own blood system (myeloablation) before this is restored through transplantation of blood cells from another mouse.

Transplantation will be performed only once in a life time of a mouse. Depending on the amount of radiation given, mice may experience post-irradiation illness but our experience with this situation will effectively limit this condition. Human blood cells may be transplanted into special mice that lack an immune system and tolerate the growth of these cells to confirm the findings from studies in mice or to identify subtle areas of difference between humans and mice.

Mice will be killed by one of the approved humane killing methods. Upon killing of a mouse, we will collect different organs (e.g. bone marrow, spleen, liver) to analyse multiple areas of interest, such as proportions of different cell types, and what genes and proteins are switched on in the cells.

What are the expected impacts and/or adverse effects for the animals during your project?

General health monitoring is performed for all mice by trained personnel, both in the animal facility and in our own research team. Given our long-standing experience with these mouse models of blood cancers, we have an in-depth understanding of the possible adverse effects of our experiments and can enhance animal care for the mice at increased risk of ill-health. In addition, when visible signs of sickness or distress are observed, enhanced monitoring and support will be put in place according to the care plans included in this license.
During our project we will use different procedures and experimental setups which are expected to have various levels of impact on animal wellbeing as listed below.

Breeding of genetically altered mice is not expected to generally cause harmful effects. Nearly all of the genetic changes used for our project will be seen in the adult animals used for our experiments, by which time they will usually have been moved to an experimental protocol. Therefore, the majority of the animals used for breeding will not experience adverse effects which might arise from changes in their blood system.

Induction treatments, such as injections of pIpC or tamoxifen which trigger switches within the mouse cells, are given to activate genetic alterations. Activating substances will be injected in mice up to 7 times, given every day or every other day. A mild weight loss up to 10% of pre-treatment weight may appear in relation to biological effects of injected substances. These adverse effects will decrease or stop once the induction period is finished and will not affect the mice long-term.

Blood collection will be used to obtain samples for tracking of normal blood formation and disease progression and other analyses. This will be performed up to 20 times during the life time of a mouse, with at least 2 weeks between the bleeds. The bleeding may lead to short-term discomfort and stress but this will be minimised by the preferable use of saphenous vein sampling and is not expected to affect the animal in the longer-term.

Blood cancer will develop in some of the mice. Over weeks or months, these mice usually show gradual changes in the number of blood cells, which may cause an increase in the size of the spleen and occasionally, in liver size. In studying lymphoid malignancies,

swelling of the lymph glands or other tumours may also occur. Their growth will be judged by external signs such as swelling of the abdomen or the appearance of masses under the skin. Occasionally testicular swelling may be apparent. Mice may appear hunched and scruffy and their hair may stand on end and they may show reduced activity towards the advanced stages of the disease. These signs will be collated and will be included in the humane endpoints.

Transplantation, a transfer of blood stem cells, will be performed in some of the mice. This is a standard method to assess the potential of cells to regenerate the whole blood system in a recipient mouse and represents a massive challenge to blood cells. The tail vein will be used as the most frequently route via which to inject the transferred cells. A transient discomfort and stress due to a temporarily handling of the animal, as required for injection, may lead to a short-term discomfort and stress-induced behaviour. However, the procedure itself is not expected to negatively affect the animals in the long term.

The injection of abnormal blood cells into the cavity of one of the long bones (e.g. thigh bone) may be used in specific cases to improve the efficiency of the transplantation or to modify the experimental outcome. This is a surgical procedure and is discussed below, under a paragraph 7 about surgeries.

Preconditioning (preparation) may be needed for some mice before they have transplantation. Irradiation will be used most frequently, but other methods may be used as well. Irradiation may induce a post-irradiation illness and the level of the illness depends on the amount of irradiation (the dose) received by a mouse. The doses will always be adjusted to ensure the least possible dose is used, therefore limiting animal suffering.

Low dose of irradiation may cause a mild drop in weight of mice (up to 10% of pre-irradiation weight). The reason for this is predominantly a decreased interest in food and water, leading to poor eating and dehydration. The activity of mice should remain unchanged. Irradiated animals will be checked daily and weighed at least twice weekly to monitor their health. An enhanced pre- and post- irradiation care plan will be provided if necessary. Our experience and the published data show that the mice recover fully from the irradiation-related adverse effects, usually within 7-10 days. The mice should return to their weights within 7-10 days post-irradiation.

High dose irradiation leads to a long-term failure of the entire blood system and therefore, the irradiation induced ill-health is more pronounced. Mice may drop in weights by up to 15% of their pre- irradiation weight. Moreover, mice may be less active. The mice should return to their weights and activity within 7-10 days post-irradiation.

Sometimes, only a part of a mouse body will be irradiated. These mice will be put under for 20-30 minutes, using an anaesthetic (inducing a state of a deep sleep). This short anaesthetic will not result in long-term adverse effects.

Preconditioning will be performed only once in the lifetime of a mouse.

Surgical procedures will be performed in aseptic conditions to prevent infection and under anaesthesia. Following surgery, mice will be kept warm until they are fully recovered and moving freely around the cage. The mice are expected to recover from anaesthesia within 20-30 minutes at most and no long-term effects are expected. Medication to relieve any pain will be administered to the mice while they are anaesthetised for the surgical procedures. Mice should be fully active within 2 hours from surgery. Surgeries should not

result in long-term adverse effects. Difficulties to regain activity beyond 2 hours post-anaesthesia or signs of more than mild pain and distress will be humane endpoints.

Imaging may be performed on some mice to visualise blood cancer growth. Injection of a substance for imaging will be performed before each session. Each session will last up to 45 minutes. At most, each animal may undergo a maximum of 15 imaging sessions in total, with no more than three imaging sessions per week Imaging will be done under anaesthesia (a state of deep sleep) and the mice are expected to be fully active within 20-30 minutes post-anaesthesia. Imaging is not expected to result in long-term adverse effects. Animals will be allowed to fully recover from anaesthesia in between sessions and will only be imaged if they are deemed to be fit enough for the imaging session.

Different treatments will be used to alter positively or negatively the progression of the blood cancers under study. We will use only substances with known properties and known to cause up to moderate adverse effects such as reduced activity, decreased feeding and weight loss up to 15% of pre-treatment weight. These adverse effects may persist throughout the whole period of treatment. The mice may receive the substances in drinking water, by specific feeding (gavage), in food or by injections.

Physiological treatments that we expect will positively or negatively alter the progression of blood cancers will be used to mimic states normally met during a human lifetime. Examples of such treatments include a nutrient-deficient (folate) diet and inflammation. These treatments may result in mild disturbances to the normal condition of an animal, such as mild changes in their activity and weight gain or loss up to 10% of their pre-treatment weight. These treatments will last up to 12 weeks.

Ageing is a physiological process and is not expected to have more than mild effects on mice. General physiological features of ageing such as reduced activity, weight change, hair loss or occasionally blindness may appear and persist until the animal is killed. The ageing mice will benefit from an enhanced care plan and be killed immediately if they experience any signs of suffering. Rarely, different cancers may develop in the oldest animals and an existing blood disease may progress faster in aged mice.

Unless otherwise specified, the work in this licence will be undertaken in accordance with the principles set out in the Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer (2010) 102, 1555-77.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Below is the summary of animal numbers used on different severity protocols: Moderate severity, 10950 mice:
Protocol 2: Embryo recipients 400 - all mice will reach moderate severity Protocol 3: Vasectomy 100 - all mice will reach moderate severity
Protocol 6: 4000 moderate - around 10% predicted to reach moderate severity (400)

Protocol 7: 5000 - 25% predicted to reach moderate severity (1250) Protocol 8: 3000 - all will reach moderate severity (3000)
Protocol 9: 4000 - all predicted to reach moderate severity (4000) Protocol 10: 4000 - 50%

predicted to reach moderate severity (2000) Mild severity, 15,400 mice:
Protocol 1: Superovulation 400 - all mice will reach mild severity

Protocol 5: Breeding 15000 (this number includes animals that will be moved to other protocols)

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 formation of blood cells is highly complex. It is not possible to fully replicate its baseline function or how this is altered during the evolution of blood cancers or how these developing cancers respond to treatment using test tube experiments in laboratories. Only by studying living models can we reach reliable conclusions and produce evidence that will permit our research to advance to studies in man.

Which non-animal alternatives did you consider for use in this project?

To answer our scientific objectives, we always utilise available data within the public domain, make extensive use of cell lines and perform non-regulated laboratory studies in the test tube on human and animal tissue that will minimise work in living animal models. With a collaborator, we have recently established mesenspheres, three-dimensional systems that include co-cultures of both blood cells (abnormal or normal) and cells derived from the bone marrow microenviroment. These better mimic the situation in animals by providing some of the external signals that we wish to study. Taken together, these strategies have already allowed us to replace some animals required for this project.

Why were they not suitable?

These experimental systems do provide useful data that supports and is complementary to experiments in living animals. However, they lack the complexity to accurately reproduce and represent the whole blood forming system of a living 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 used the experience of our previous projects to estimate the numbers of animals required. These are also based on the predicted growth in size of our respective groups and the shift to perform more translational/therapeutic experiments. This project has a moderately increased scope on our previous licence, including increased complementary work on lymphoid malignancies and a greater focus on the earlier stages of malignancy development where the changes are more subtle and larger numbers of mice may be necessary. Therefore we anticipate a slightly greater number of experiments. However, the measures we will take, as described below will allow us to reduce the average number of animals required per experiment. Our estimated numbers are thus substantially in line with our previous Project Licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have employed several advances in practice, including the NC3Rs experimental design assistant, in designing our experiments which have allowed us to reduce the number of animals being used in this project.

- Tissue collection: Previous general practice has been to harvest bone marrow from the long bones of the lower limbs of mice. This typically provides around 10-40 million cells per mouse. We will be employing a newer methodology that uses almost the whole skeleton to provide bone marrow and can yield 100-400 million cells per mouse. This will allow us to reduce the number of mice that we need to use for such tissue-based experiments by approximately 50%.
- Bioluminescence: The main method previously used to assess tumour growth kinetics was white blood cell count. Adopting bioluminescence as a method of assessing tumour growth will allow this to be performed with greater accuracy, reliability and efficiency. Since development of disease can be investigated longitudinally, the use of imaging methods will also allow a reduction of numbers of mice required. We will genetically alter tumours to express the luciferase gene prior to transplantation. Administration of a substance, luciferin, is used by luciferase to emit light from the luciferase containing cells, that can be detected by sensitive cameras. The light emitted is therefore directly proportional to the number of cells that contain luciferase and to the tumour size. The greater accuracy and reliability with which we can assess therapeutics and other alterations that validate potential therapeutic targets has also allowed us to reduce the number of mice required.
- Increased use of injections directly into the long-bones of the animal to generate bone marrow transplant recipient. This will result in a more efficient and reproducible process and will therefore require fewer donor animals.
- Experiments have been designed based on previously published studies as well as current manuscripts, grants and ARRIVE guidelines. Published studies and grant awards have been thoroughly peer reviewed, including by statisticians, to help ensure numbers of animals are reduced as far as 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 will take all possible measures to optimise the number of animals on our project. Examples include: Sharing tissue within our institution.
- Collaborating with other researchers and consulting published data to avoid strain and experimental duplication.
- Employing pilot studies with small numbers of animals to optimise experiments and

therefore reduce the total numbers of animals required to answer a specific research question.
- Employing the most efficient breeding techniques, including timing, rotation and good husbandry.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 project uses mice as a model of the blood forming system and of blood cancer formation. The mouse is the most appropriate and most widely used model in this field. The methodologies used are very well established and have been refined to cause the minimum pain, suffering, distress and harm to the animals. An example of this is saphenous vein sampling rather than tail vein sampling to provide blood samples. The saphenous vein is larger than the vessels in the tail and can actually be readily punctured for sampling, as in a human blood test from a large vein in the arm, rather than tail vein bleeding where the blood seeps out as it would from an actual wound. The majority of the protocols have mild impact on the animals. Protocols which have a greater impact on the animals are only used when there is no alternative. We will also make use of analgesia on all procedures where this is readily available and appropriate (e.g. intrafemoral injections).

Why can’t you use animals that are less sentient?

Adult mice are used in our project as the least sentient species from which meaningful experimental data can be generated. They share sufficient physiological and pathological traits with humans and yet are less sentient than other mammalian models. Because mice are the most appropriate and most widely used model, our data can be compared with other researchers', increasing the value of our studies to the scientific community as a whole. As blood cancers often require extended periods of time to manifest, it is not applicable or appropriate to use terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will follow best practice as established locally and follow national guidance by e.g. NC3Rs to minimise the welfare costs to our animals. Examples of best practice we will employ include:

- Anaesthetic and pain medication will be applied when animals undergo surgical procedures.
- Needles will only be used once. This reduces infections sustained by animals and ensures that only sharp needles, which cause less pain, are used.
- Oral gavage, a procedure whereby blunt-ended needles are used to introduce controlled volumes of liquids into the stomachs of mice, mimicking oral administration

of drugs, will be used to administer potential therapeutic agents to remove the need for injections.
- A bioluminescence methodology whereby tumours are genetically altered to express the luciferase gene prior to transplantation. This allows the accurate assessment of tumour growth kinetics by non-invasive imaging in living animals, removing the requirement for blood sampling and the greater handling required for clinical assessment.
- Nail clipping will be employed on mice exhibiting skin irritation to prevent overgrooming and scratch wounds.
- Facilities with special restrictions preventing the introduction of infection will be used to protect the health of immunosuppressed mice.
- Appropriate dosages will be calculated following extensive experimentation on cell lines.
- The health of all animals will be monitored at least daily, with particularly close monitoring of immunosuppressed mice for the development of opportunist infection.
- Whenever possible (e.g. in haematological malignancies that present with distinct and consistent peripheral blood abnormalities), serial blood counts will be used to predict endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow established best practice guidance in planning and carrying out our experiments, re- visiting the guidance as it is updated. In particular we will follow the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines produced by NORECOPA and the Guidelines for the Welfare and Use of Animals in Cancer Research produced by the National Cancer Research Institute (Workman et al, British Journal of Cancer, 2010) and the LASA guiding principles https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf). We will also follow the ARRIVE guidelines when planning and reporting our animal studies (https://arriveguidelines.org/arrive-guidelines).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Animals at our establishment are cared for by a team of highly-trained professional animal technicians who regularly review and update their practice to include advances in the 3Rs. Advances in 3Rs are communicated through user group meetings and written communication. Furthermore, we will seek out information on advances in practice from national sources such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (https://nc3rs.org.uk/the-3rs). In particular, we will take advantage of their webinars, e-learning and other online resources. We will also make use of international resources, such as the Humane Endpoints website of the 3Rs Centre at Utrecht University. (https://www.uu.nl/en/organisation/3rs-centre) and Norecopa, Norway's 3R centre and National Consensus Platform for the Replacement, Reduction and Refinement of animal experiments (https://norecopa.no).

191. Understanding the regulation of fat mass and its association with 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

Metabolism, Adipose Tissue, Obesity, Type 2 diabetes, Insulin sensitivity

Animal types	Life stages
Mice	adult, neonate, juvenile, 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 define the biological function of genes linked to human fat mass regulation and metabolic disease and in some instances, leading to the identification of novel therapeutic 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?

Both too much (obesity) and too little (lipodystrophy) fat are strongly associated with human metabolic disease e.g. type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). Considerable progress has been made in understanding the genetic factors which cause obesity and lipodystrophy in humans but in many cases the function of the

relevant genes remains unclear. Mice have proven to be very valuable animals for advancing understanding of the biological function of the genes identified. Ultimately this work has and may yet lead to the discovery of novel therapeutic targets for human metabolic disease.

What outputs do you think you will see at the end of this project?

We will characterise the body weight/ fat mass and fat distribution of genetically altered mice. These mice will be designed to provide novel information on genes implicated in human obesity.

We will then document changes in weight and fat mass of these mice in response to a regular or high fat diet in an attempt to assess their capacity to adapt to nutritional challenges.

In cases where the genetic changes do alter weight and/or fat mass, we will also document the impact of these changes on carbohydrate/ fat metabolism and on cold temperature tolerance, providing new information on how the genes under investigation impact human metabolism and health.

This work will be presented at major scientific meetings around the world and will be published in scientific journals.

Who or what will benefit from these outputs, and how?

The annual UK-wide NHS costs attributable to overweight and obesity are proposed to reach £9.7 billion by 2050. Most of this cost relates to dealing with the metabolic diseases associated with obesity; including type 2 diabetes, fatty liver disease and cardiovascular disease. Interestingly, lipodystrophies, which are diseases characterised by a pathological lack of body fat, are also associated with these metabolic diseases. So both too much and too little fat can lead to serious metabolic disease but why this is so, remains incompletely understood.

The generation and characterisation of mice in which the expression of genes we know are linked to human metabolic disease through an effect on fat mass will help us to generate new insights into the causes of type 2 diabetes and other common metabolic diseases, and to potentially reveal novel therapeutic drug targets.

In the short term, the scientific community will gain new information on the genes which we know will be of interest as they will be selected based on being associated with human weight and fat mass.

In some instances, the molecules under investigation may themselves be treatment targets or indirectly inform new treatment strategies for common metabolic problems such as obesity, type 2 diabetes and fatty liver disease.

The knowledge we generate may also help patients with rare but very serious metabolic problems such as lipodystrophy.

How will you look to maximise the outputs of this work?

We already collaborate with several other groups studying metabolic disease around the world, and will publish our results in scientific open access journals. We will also present

the findings at scientific meetings as posters and oral presentations. This typically applies to positive and negative findings as all our work relates to novel genetic models so we simply describe what we observe either way - it is not really 'positive' or 'negative' in the same way that a trial of a new treatment would be.

Species and numbers of animals expected to be used

- Mice: 8470

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The purpose of this project is to understand the biological function of genes linked to human fat mass regulation and metabolic disease. Mice have been used for decades to understand how genetic alterations cause obesity and why genetically altered mice prone to obesity may develop type 2 diabetes, so are well-established animals for this type of project. Insulin action is a key factor in the link between obesity and diabetes. Its complex actions on several different organs requires whole animals in order to study how all the different organs and systems in the body work together.

The project is focussed on changes in fat mass and metabolism which typically only manifest after weaning in mice so we will focus on juveniles and adult mice.

Typically, what will be done to an animal used in your project?

Firstly we will use established standardised breeding protocols to generate genetically altered mice.

These mice would then be group housed in their home cages while being supplied with a regular diet or a high fat diet for 24 weeks. Occasionally mice may be singly housed (for up to 48 hours) in order for us to accurately determine how much food a single mouse is eating or to determine its metabolic rate. We might also singly house mice (for up to 7 days) whilst reducing their usual calorie intake by up to 30%. Alternatively we might undertake a similar calorie restriction for a more prolonged period (up to 3 months) in group housed mice. During this time they would be weighed on a weekly basis and blood samples would be taken to check glucose and insulin levels no more than 4 weekly, unless a mouse showed signs of diabetes (drinking excessively/ passing excess urine). We would also assess fat mass regularly using time domain nuclear magnetic resonance (TD-NMR), which is a piece of equipment used to measure body fat in the awake mouse. Then towards the end of the study we might perform a glucose tolerance test using an intraperitoneal (this means into the abdominal cavity) glucose injection. After recovery for one week, the mouse might then have an insulin tolerance test (intraperitoneal injection again) before being humanely killed for tissue collection and final blood collection. Before the glucose and insulin tolerance tests mice will be fasted for up to 6 hours.

What are the expected impacts and/or adverse effects for the animals during your project?

Our studies will involve the following:

Blood sampling with its associated handling stress and transient discomfort.

Intraperitoneal injections with their associated handling stress and transient discomfort.

Some mice may develop diabetes which will result in increased drinking and urine production. If this is noted we will monitor blood sugar levels and if they go too high the animal will be killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

Mild: over 90% Moderate: less than 10%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals as diseases like diabetes involve interactions between several key organs like the fat, liver and muscle. Many of the questions we are interested in can only be studied in complex organisms like mice, rather than simpler organisms like flies, worms or fish which don't have fat under their skin as humans and other mammals do. Where possible we do and will study more basic questions in cells, yeast or flies, but none of these has true fat tissue so they are inadequate to really address the role of the genes/proteins we are interested in.

Which non-animal alternatives did you consider for use in this project?
We perform some studies using tissues removed from humanely killed mice, such as using adipose (fat) tissue explant (tissue removed from a dead mouse and then maintained in a fluid bath) assays that allow us to analyse some of the tissue functions whilst minimising suffering of the mice.

We also use primary cells isolated from the mice, like Mouse Embryonic Fibroblasts (MEFs) or Ear Mesenchymal Stem Cells (EMSCs), or well established cell lines to perform experiments in cells grown in the laboratory. We have used MEFs or genetically engineered cells, to enable us to work with cell lines representative of the mouse strains, thereby reducing animal numbers.

Why were they not suitable?

For metabolic studies we need to be able to use a system which involves interactions between different organs; specifically fat, liver and muscle as these are all key organs in insulin action, and impaired insulin action is a major factor in causing type 2 diabetes. Cells and animals, such as insects and worms, do not have body systems that work in the same way they do in humans and 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?

We have conducted very similar studies to those proposed herein in the past, so have based our current estimates on this prior experience.

We also work in an institute focused on understanding metabolism so have several colleagues with overlapping interests. Our institute also has a core metabolic phenotyping team who ensure that expertise is shared across the institute and provide support and advice to each team. So again relevant shared experience has been used to estimate our numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A key aspect of reduction involves studying only carefully selected strains of mice. My research programme does not involve large-scale use of genetically altered mice but instead focuses on very specific questions which cannot be addressed in humans or other simpler organisms.

The methods we use to characterise mice have been used extensively within my group so we have the necessary expertise to work out the minimum number of mice needed to answer the questions we are working on. Typically we would assess a new mouse strain in a group size of 8-10 mice on a basic protocol.

Non-invasive technology: Use of sequential non-invasive methods for phenotyping means that we can generate data without killing the mice, thus minimizing the number of mice needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding: This will be done by experienced staff who work for the core facility in our institute. We will also obtain animals from relevant suppliers where these exist rather than generating lines ourselves, thus minimizing breeding.

Our work is guided by the NC3R's PREPARE (Planning Research and Experimental

Procedures on Animals: Recommendations for Excellence), the ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines, and the UK Home Office "Assessment Framework" on the efficient breeding of genetically altered animals (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf).

We write comprehensive Study Plans for each experiment which include:

a statement of the experiment objective(s)

a description of the experiments, covering topics such as proposed diets, the size of the experiment (number of groups, number of animals in each group), and the experimental materials to be used

an outline of the method of analysis of the results is included (which may include the planned statistical 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.

We will only be using mice where information from human studies has not already led to comprehensive understanding of the function or impact of a gene or gene variant, and where a simpler cell based or model organism cannot be used.

Genetically altered/modified mice that develop obesity and diabetes have proven for decades to be valuable in helping us in this type of research and so are a particularly suitable animal for our experiments.
All of the genetically altered mice will be used in tried and tested procedures. We are not expecting the procedures (e.g. blood sampling and injecting drugs) and the majority of genetic alterations made to cause more than mild and transient discomfort which means our animals are expected to remain healthy as well.

Why can’t you use animals that are less sentient?

Obesity typically causes diabetes by impairing insulin action. Insulin acts on several organs including fat, the liver and muscle so we need an organism with these organs and in which insulin action can be evaluated in each of these organs - this is not possible in simpler organisms. My laboratory has experience with cells, yeast and insects so is well placed to know when these can and cannot be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To improve the quality of life of the animals we:

reduce stress by group housing where possible to keep singly housed mice to a minimum.

use environmental enrichment (EE), within what is available to us at our animal facility. In general, EE is an animal housing technique composed of increased space, physical activity, and social interactions, which in turn increases sensory, mental, motor, and social stimulation. Igloos, running wheels, saucer wheels, fun tunnels, and other objects in the housing environment provide stimulation by promoting exploration and interaction. EE can be maintained when animals are handled (e.g. handling tunnels), thus minimizing stress when for example an injection is needed.

use a series of non-invasive methods for characterising mice so that we can generate useful data without killing the animals, thus minimising the number of mice needed

use pain killers to lessen pain.

provide 'behavioural' training to mice undergoing specific procedures (e.g. acclimatization to single housing in cages used to measure food intake and energy burned).

use scoring sheets to monitor the health of animals undergoing procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique (https://www.lasa.co.uk/current_publications/)

ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (https://www.nc3rs.org.uk/arrive-guidelines)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning our experiments (15 topics including formulation of the study, dialogue between scientists and the animal facility, and methods) (https://www.ncbi.nlm.nih.gov/pubmed/28771074).

The UK Home Office "Assessment Framework" on the efficient breeding of genetically altered animals (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To be informed about latest advances we will primarily use the National Centre for the Replacement and Reduction of Animals in Research (NC3R) website (https://www.nc3rs.org.uk). It provides an extensive library of 3Rs guidelines, resources, practical information and themed hubs. It also provides links to publications, other online resources, and video and training materials.

Implementation of the advances will be defined on a case-by-case basis, and will be informed by the latest NC3R recommendations.

We also share expertise across our institute with regular internal seminars and have an external seminar series so hear from other experts on a regular basis.

We have a team of researchers at our institute who specialise in using mice in obesity and diabetes research and they regularly provide us with updates on new advances.

Home Office

192. Breeding and maintenance of genetically altered zebrafish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Embryos, GA lines, Cryopreservation

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This licence will provide breeding authority for users who only require pre-protected stage zebrafish larvae or require to perform post-mortem tissue analysis. All protected animals generated under this will be solely to maintain transgenic lines and to produce the required embryos/larvae.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 large proportion of zebrafish research at the Establishment occurs on pre-protected larval stages, i.e. before they become capable of independent feeding- usually 5 days post fertilisation. Performing experimental procedures on pre-protected stage embryos &/or larvae does not require a Project Licence, but in order to produce and maintain the adult animals which generate the genetically altered embryos &/or larvae for this, a Project Licence is required. This service licence will bring all the individual fish research groups together under one central umbrella. This, in turn, will create centralised expertise and allow for optimisation of the 3Rs.

What outputs do you think you will see at the end of this project?

New transgenic zebrafish lines, publications arising from investigations on pre-feeding stage larvae, and adults for post-mortem tissue analysis.

Who or what will benefit from these outputs, and how?

In the immediate term, researchers will be able to investigate physiological processes and diseases in non-protected larvae, and to use adult tissue after they have been killed humanely by an approved and appropriate method. In the medium term, new transgenic lines will be created to investigate the above processes. It is difficult to quantify the long-term benefit of this programme other than advancing our understanding of physiological and disease processes.

The fish themselves will benefit from a centralised PPL as it will ensure high standards of welfare are maintained throughout their life experience, whilst enabling improvement to care to be implemented more readily.

How will you look to maximise the outputs of this work?

All transgenic lines created and maintained under this licence will be available to other researchers, provided appropriate justification is provided.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 111000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 licence is intended to act as a service licence, in which users are able to create and maintain genetically altered zebrafish lines. To do this, it is imperative that all life stages are included.

Users will also be able to obtain life stages that are not covered by the Animal (Scientific Procedures) Act (pre-protected stages are zebrafish at less than 5 days post fertilisation where they are not capable of independent feeding) for their scientific studies. This reduces the need to use later stages that are more sensitive to experiencing a level of pain, suffering, distress or lasting harm.

It also affords them the opportunity to cryopreserve their lines, in which case adult zebrafish would be needed.

Typically, what will be done to an animal used in your project?

We will produce create, breed and maintain transgenic lines of zebrafish or produce adults that will be humanely euthanized to provide tissue for experimental analysis.

What are the expected impacts and/or adverse effects for the animals during your

project?

The only painful procedures to be conducted that will have a lasting impact are fin biopsy to obtain genetic material for genotyping. In these cases, the fin regenerates within 14 days with no lasting welfare impact. We will use post-operative analgesia to minimise any pain and suffering in these circumstances.

Expected severity categories and the 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 nature of the genetic alteration should not impact animal welfare to more than a mild amount.

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?

In order to breed zebrafish larvae, adult animals are required. Zebrafish larvae will be used to investigate the interaction between individual cells and molecules in a vertebrate non-protected animal model.

Which non-animal alternatives did you consider for use in this project?

The physiological interactions will be investigated in in vitro as far as possible.

Why were they not suitable?

Whilst useful in establishing simple interactions in vitro, our models are not yet suitably advanced to allow the full interactions to be modelled and predicted with certainty in an ex vivo setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals we predict to use is based upon feedback for our researchers of the numbers of larvae they predict to use and the number of parents we would require to breed to create the required number of larvae.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Given that the outputs from this service licence will not be numerical data, experimental design calculations have not been possible. However, the design of our breeding strategies will be dictated by user demand and will be rigorously reviewed during the lifetime of 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?

All breeding under this licence will be stringently managed by highly experienced technicians. Careful management of the fish lines will help to reduce the number of animals produced; i.e. animals will only be produced where there is a justifiable need. Where possible, all larvae bred will be shared between research groups and will only be dictated by the genetic alteration of the strains.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 zebrafish will either be created or maintained under this licence. Only genetic alteration that at most are predicted to cause mild impact on animal welfare will be bred under this licence. Where a new genetic alteration is made, the resulting progeny will be monitored carefully during gestation and the non-protected phases to identify any mutations that could potentially impact on animal welfare. These animals will be additionally carefully assessed for any welfare impacts once they reach free-feeding stages for any suffering. Any animals found to be suffering more than mildly will be euthanized humanely and the breeding of those animals re-assessed.

Why can’t you use animals that are less sentient?

We will principally use immature zebrafish larvae and the only protected animals we will produce will be for maintenance of the transgenic line or to produce adults that will be euthanized to provide tissue for experimental analysis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where animals will be subject to painful procedures, for example fin biopsy to obtain genetic material for genotyping, we will use post-operative analgesia to minimise any pain and suffering. We are also exploring alternative methods such as the use of the ZEG machine that allows for the genotyping of embryos, prior to the point they become protected.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult with leaders in the field of zebrafish breeding including the NC3Rs to implement any improvements in animal welfare.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult with leaders in the field of zebrafish breeding including the NC3Rs to implement any improvements in animal welfare and non-animal alternatives.

193. Modelling and preventing tumour-bone marrow interactions in metastasis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Multiple myeloma, Metastatic cancer, Dormant tumour cells, Drug resistance, Bone disease

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?

This project seeks to further understand the regulatory systems that control tumour cell interactions within the bone marrow microenvironment and to identifying new approaches to regulate their activity in preclinical models of 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?

Tumours that grow in bone remain incurable for most cancer patients; and exactly how tumour cells spread to the skeleton in patients with breast cancer, prostate cancer and multiple myeloma is still not fully known. Despite significant improvements in drug treatments, some tumour cells can still remain in bone leading to disease relapse and significant bone damage. Therefore, we aim to gain a better understanding of how cancer

cells spread to bone (bone metastasis) so we can develop new treatments or modify the delivery of existing treatments to kill all tumour cells. In addition, current treatments of bone disease caused by tumour growth are restricted to therapies which prevent, but do not repair, existing bone damage. Therefore, in this project, we aim to identify key factors that cause tumour cell spread and survival in the skeleton and identify existing or new drugs to repair bone disease. Overall, this new knowledge will lead to improved treatments for patients with tumours that grow in bone.

What outputs do you think you will see at the end of this project?

At the end of this project we will have acquired new information on what (factors, pathways) controls cancer cell spread and growth in bone and how we can modulate these to prevent disease progression. We will disseminate this information at scientific meetings (local, national and international) and by publishing our data in high impact peer-reviewed scientific journals.

Who or what will benefit from these outputs, and how?

Our findings will lead to new treatments or the modification of existing treatment regimens for patients with cancer-induced bone disease. This will initially require the use of animal models, but promising approaches will be rapidly trialled in patient cohorts. This would be a very important advance in the treatment of multiple myeloma, breast and prostate cancers as earlier diagnosis in all these diseases offers the opportunity to intervene at earlier stages and prevent disease progression. It is anticipated that translational benefits in patients would be seen within 5 years as has already been the case with developments in the use of bisphosphonates (bone modulating drugs) in the treatment of cancer-induced bone disease that resulted from our own work and that of others.

How will you look to maximise the outputs of this work?

We will collaborate with basic scientists and clinicians locally and at other research institutes (UK and worldwide). The results generated form this project would be disseminated at highly relevant scientific meetings (EHA, ASH, UKMF) and published in high impact factor journals.

Species and numbers of animals expected to be used

- Mice: 3200 animals maximum over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
We will use established mouse models of cancer (breast, prostate, myeloma etc) where we know tumour take rates are high and disease (tumour growth and bone disease) variability is relatively low. The age of mice at the start of each study will be 5-10 weeks old and at sacrifice >12 weeks old which will be dependent on the specific cancer model.

Typically, what will be done to an animal used in your project?

Mice will be injected with tumour cells at the start of each study and disease progression (tumour and bone) will be monitored by imaging methods. Intervention therapies will be assessed at different stages of disease progression by imaging or mice may be killed at specific time points depending on the research question been addressed.

What are the expected impacts and/or adverse effects for the animals during your project?

General signs of ill health will be assessed throughout each study using the principles of the grimace scale for mice.

Potential adverse effects for tumour growth and metastasis are weight loss (>20%), hypothermia, enlarged lymph glands, bleeding/mucopurulent discharge from any orifice, anaemia, incontinence/diarrhoea, hind limb paralysis/weakness, poor locomotion, hunching and/or tremors or convulsions or disturbed breathing, and other signs of pain. If any of these signs are observed animals will be killed immediately.

No adverse effects are expected for evaluation of pharmaceutical agents other than the possibility of transient discomfort where injectable routes, or gavage are used and following repeated doses.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for all tumour models and for the evaluation of pharmaceutical agents will be moderate. For collection of mononuclear cells it will be 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?

The complexity of the interactions between tumour cells and bone resident populations is such that it would not be possible to model the ‘metastatic niche’ and "repair of established bone disease" in its entirety using cell culture. The behaviour of cells within the niche and bone is both interactive, dependent on signalling between cells but is also highly dependent on the presence of the bone matrix and systemic factors that control and modify this. In some specific cases it may be possible to model simple interactions between two particular cell populations in vitro and this will be done where possible to explore hypotheses related to interactions and to replace animal experimentation.

Which non-animal alternatives did you consider for use in this project?

All pharmacological agents/drugs will be initially assessed in vitro on tumour cells (proliferation/dormancy assays) and bone cells (alkaline phosphatase, mineralisation assays) before progression into animal models.

We are also investigating a non-animal alternative system for assessing drug effects on dormant cancer cells.

Why were they not suitable?

Bone metastasis is a complex disease with multiple effects on a variety of cells in the bone marrow microenvironment (BMME) as well as changes in the structure of bone itself. Unfortunately, it is not possible to accurately mimic these complex conditions of the BMME in cell culture due to the presence of many different cell types that physically interact and signal between each other, as well as the presence of a bone matrix which produces various systemic factors that controls and modifies the BMME. As a result, in vitro testing does not provide complete mechanistic data for the role of a particular drug and also doesn't account for any systemic effects in animals. Therefore, we must rely on data obtained from well-designed in vivo experiments to determine the true efficacy of drugs on tumour burden and bone disease before translation 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?

Protocols are based on previous studies where efficient statistical differences have been demonstrated. In studies of tumour bone interactions, 8-12 animals/treatment group (dependent on mouse model) will provide at least 80% power to detect differences using analysis of variance (data based on references in the Project plan section). For assessments of the pharmacological agents, animal numbers will be limited to 6/treatment group as experience indicates that there is less variability in these types of studies. Routine passaging of cells will be restricted to the minimum numbers of animals required to maintain the cell line. Normally tumour from one animal will be passaged into 4 recipients/generation and where possible frozen stocks maintained rather than growing populations in animals. We will undertake to revisit the assessment of animal numbers required to complete these studies in the light of new developments in technology. In particular, improved in vivo tumour and bone imaging has enabled us to undertake longitudinal experiments with repeated measurements in the same animals rather than having to kill cohorts to obtain the same endpoints (i.e. tumour burden, bone parameters) ex vivo.
Advances in these areas are likely to improve experimental design, and statistical advice will sought throughout the study to take new developments into account and reduce animal numbers.

As part of good laboratory practice, we will use the NC3Rs Experimental Design Assistant (EDA) and write standardised protocols for each experiment including - objective(s); materials/methods/experimental plan (animal numbers/groups and schedules) plus an

outline of the methods that will be used for the analyses of results (statistics etc). While the overall numbers of animals may appear large, the licence covers the activity of 3 interconnected research groups. Under Protocol 1, 2500 animals over 5 years is 500/year for all projects. Bearing in mind that each study would have a minimum of 8-12 animals/treatment group and so that simply testing one agent vs controls would be 16-24 animals to do the experiment once. With 3 biological repeats this number would rise to 75 – this is just one simple experiment for one research group. Add combinations or more than one treatment time point and these numbers rise rapidly. The numbers calculated are based on the last 5 years' experience of experiments done by the research groups whose work was carried out under a previous project licence and is a maximum estimate. Similarly, the other estimates are maxima based on past experience.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use the NC3Rs EDA.

In undertaking this type of study, we will undertake to revisit the assessment of animal numbers required to complete these studies in the light of new developments in technology i.e. more sensitive methods to monitor tumour load and bone disease. Therefore, statistical advice will be sought throughout the programme of work (from a statistician who specifically supports animal experimenters) to take new developments into account and reduce animal numbers. In addition, we will be applying tried and tested analyses to maximise data collection from each animal.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will perform pilot studies before we progress onto major studies to ensure all methods are completely established.

We are in continual discussion with computer modellers to assess if some studies can be digitally modelled (tumour and bone progression with and without drug interventions) rather than using animals.
We will make tissue available to other researchers locally and with our collaborators where possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models

We will use two general types of mouse models of cancer to study the effects of new or

existing cancer therapies. The first model uses human tumour cells or tissue implanted into mice that lack part of their immune system. This is required so that the body does not reject the human tumour cells. The second model uses mouse tumours implanted into mice with a fully functioning immune system. This is also important as the immune system can influence tumour growth, bone disease and response to therapy. These models have been extensively used by us and others, so there is a wealth of information available on these models and they are considered to be reliable and reproducible. We will plan experiments to not let tumours grow to a size where they cause the animal to become significantly unwell, we will use earlier timepoints at which the animals will be humanely killed.

Methods

We will also use general practices that minimise stress, harm and pain to animals. This includes non- aversive methods for handling mice (e.g. not picking up by the tail), the use of single-use needles to avoid pain from dulled needles and NC3R guidelines on blood sampling.

For tumour cell injection we will do this via the tail vein for myeloma models. For prostate and breast cancer models we have gained experience with intracardiac injection via close collaborations. This technique is now so effective locally and the mortality rate for the procedure is well below 1%, with the experience of the group extending to >1000 animals. We have considered other routes to introduce tumour cells into the circulation, including injection into smaller vessels such as the femoral artery.
These are technically very challenging given the small diameter of vessels and their susceptibility to damage as well as the necessity for additional surgery to access these structures with additional complications. Our collaborators have found that the intracardiac route as described above is robust and minimizes tissue damage, which is why it is widely used for the introduction of tumour cells into the circulation.

At all stages of disease progression, we will image tumour arrival and growth by bioluminescence (IVIS) and bone disease by x-ray (in vivo micro-CT). These studies will be done with and without therapeutic intervention. We will also examine the effects of altered bone structure induced by alterations in skeletal loading on tumour colonisation and growth by non-invasive mechanical loading (of tibia or femur), running on a treadmill or standing on a vibrating platform. Procedures are mild and it is not anticipated these will have adverse effects.

Why can’t you use animals that are less sentient?

To study effect of treatments on tumour growth, tumour spread to bone (metastasis) and relapse requires a whole organism model. Mouse models have been extensively used by us and others, so there is a wealth of information available on these models and they are considered to be reliable and reproducible. Mice are also very similar to humans genetically, and many processes in relation to development and growth of cancer are conserved between these species. Mice that lack part of the immune system can also grow human tumours, allowing us to study the effect of new treatments on human tumour cells.

Some less-sentient species, such as zebrafish, can be used to study specific tumorigenic processes. However, these models cannot be used for studying the effects on intervention on processes like metastasis and bone disease. They also do not provide the ability to

model treatment strategies that are used for patients. For our studies, in order to determine whether treatments are effective at treating growth of primary tumours and/or metastasis and bone disease, mice are the most reliable model and we cannot currently perform these experiments in a less-sentient species. We find mouse models of cancer are most reliable and representative in adult mice and they cannot be used at a life stage where mice are less-sentient.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Tumours will be regularly monitored through non-invasive methods. General signs of ill health will be assessed throughout each study using the principles of the grimace scale for mice. Mice will be regularly checked for signs of being unwell, for example changes to the coat condition, behaviour, loss of weight and movement. We will also continually attempt to identify earlier endpoints for studies, to minimise harm to animals.

Invasive surgeries will be performed under general anaesthesia. Mice will be given pain-relief to manage post-operative pain and kept warm while they recover. Mice will be monitored following surgery and any procedures that may have immediate adverse effects.

To minimise harm to animals, we previously developed refined mouse models of cancer, that have minimised harm, stress, suffering and pain to mice by identifying earlier endpoints that to not rely on the animals showing signs of being unwell. This was made possible through specialised imaging techniques that can detect the disease at much earlier stages. This also made monitoring therapeutic intervention more accurate and allowed further refinement of one model to mimic a specific disease state that occurs in many cancer patients. This is something that we implement as much as possible in all models of cancer.

We will use specialised, non-invasive tumour imaging techniques under anaesthesia, which allow us to identify and implement study endpoints that do not rely on animals showing signs of being sick, in distress or in pain, while also reducing the numbers of mice required.

Only animals deemed to be in good health will be used in experiments. Animals with a compromised immune system will be housed in ventilated cages through a biosecurity barrier to minimise their risk of infection. Any techniques that may be painful or distressing to animals will be performed with anaesthesia and appropriate pain relief will be given to minimise pain (e.g. after surgery). All surgeries will be performed under sterile conditions following best practice guidelines (e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery). Animals will be regularly monitored for possible adverse effects that may occur in response to the tumour, procedures or treatments. We will seek advice from the NVS and/or NACWO where appropriate (e.g. for administration of an antibiotic if an infection is suspected. Optimal treatment regimens (e.g. dose, frequency and route of administration) will be identified in small pilot studies before use in larger scale studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All surgeries will be performed under sterile conditions following best practice guidelines, e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

Procedures for establishment and monitoring of tumour growth, metastasis and animal welfare will follow best practice guidelines (e.g. NCRI Guidelines for the welfare and use of animals in cancer research, Workman et al. 2010 Br. J. Cancer). We will also consult the NC3Rs website and Norecopa for information on the 3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly consult the NC3Rs online resources and the NC3Rs regional programme manager. I also sit on the local Project Applications and Amendments Committee (PAAC) and the Animal Welfare Ethical Review Body (AWERB).

We will also attend local, national and international workshops and conferences; and read current peer- reviewed literature to stay informed of new advances. Therefore, where possible we can use alternative non-animal model systems and improve our in vivo imaging (to visualise arrival/early stages of tumour cells in the bone and detect early signs of bone disease).

194. In vivo acaricidal activity of novel acaricide products to control poultry red mite (Dermanyssus gallinae)

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - (iii) Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Poultry, Acaricide, Red mite (Dermanyssus gallinae), Ectoparasite

Animal types	Life stages
Domestic Chickens (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?

This project aims to identify and test novel products to control infestations of the poultry red mite (Dermanyssus gallinae), by evaluating in chickens acaricidal activity (e.g. ability to kill mites) of new acaricidal formulations, under conditions that mimic commercial egg production.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Poultry red mite is the most important parasite of poultry in Europe. It is a blood-sucking parasite that feeds on the bird during periods of darkness. Poultry red mite infestation is a particular problem in laying hens that produce eggs for human consumption, where mites

disrupt normal behaviour by causing irritation and restlessness in flocks as the mites feed. Hens can also become anaemic (i.e. too few red blood cells), and even die due to severe blood loss in heavy mite infestations. Mites may also spread diseases such as Salmonella, to both hens and people working on the farm. Egg production and quality are also reduced as a result of infestation, and more of the eggs produced are rejected because of unsightly surface contamination with mites. Acaricides (a substance that kills mites) are the primary means of controlling poultry red mite. Still, relatively few products are available to legally treat infestations, with mite resistance being an ever-increasing issue for the sustained effectiveness of current chemical control programmes that rely on a small number of active ingredients.

What outputs do you think you will see at the end of this project?

Poultry red mite is the most serious parasite of poultry in Europe, affecting both the production and welfare of approximately 400 million laying hens. Control of poultry red mite, primarily achieved through artificial acaricides (a substance that kills mites), is increasingly difficult for a number of reasons so that commercial poultry producers urgently seek alternative forms of control. Under this Licence, any work to demonstrate the effectiveness of novel acaricides which can control poultry red mite offers the possibility to make a significant improvement to controlling poultry red mite, and thus benefit the health and well-being of a large number of laying hens across the UK, Europe and the globe. In generating new information on the effectiveness of novel products, data may also be obtained that are suitable for publication, as has been the case in preceding work from our group.

Who or what will benefit from these outputs, and how?

Better control of a significant pest of laying hens leading to improved farm animal welfare is the primary focus of work planned under this Licence and a crucial societal objective. Other indirect societal benefits will accrue from effective control of poultry red mite, namely a reduced environmental impact of poultry production through improved productivity of hens, greater efficiency of resource use and reduced hen mortality. Economic sustainability of food production will also be improved through a combination of reduced inputs and increased outputs, contributing to a lower cost of production.

Working conditions for farm staff might also be improved, since poultry red mite have been proposed as an occupational hazard.

How will you look to maximise the outputs of this work?

We will look to maximise outputs by promoting the work to potential commercial end-users and other possible collaborators, such as other academic institutions. Where appropriate, we will publish the results of work undertaken under this Licence in scientific journals and industry literature, further disseminating results through the presentation at appropriate industry and academic events. Whilst such public dissemination is more likely to result from publicly-funded projects undertaken under this Licence, experience has shown an appetite for publication of results from robust commercially-funded research undertaken by our group in this area, such as the widespread scientific interest in finding novel poultry red mite control strategies.

Species and numbers of animals expected to be used

- Domestic fowl: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to provide effectiveness data to evaluate the commercial potential of novel control products, studies must be undertaken under conditions that represent use under standard industry practice. In the absence of reliable non-animal feeding devices, testing the effectiveness in chickens of poultry red mite control products needs to be undertaken on live animals under the commercial conditions in which the mites pose a threat, namely in facilities that mimic commercial practice for laying hens. For our initial project on laying hens, entails using adult birds that typically experience poultry red mite infestation when entering the laying farm at 16 weeks of age and thereafter for the duration of the normal period of egg production.

Typically, what will be done to an animal used in your project?

Typically, birds will be confined in small groups in an experimental facility and exposed to poultry red mite at infestation levels which simulate those of commercial conditions. Depending upon the novel product(s) being tested, birds will then be subjected to a single application of an acaricide (a substance that kills mites). After exposure to poultry red mites for an appropriate length of time, typically 28 days pre-treatment and a further 28 days post-treatment, the study will end and the birds will be killed or rehomed if appropriate.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals subject to poultry red mite infestation may experience mild discomfort associated with the periodic feeding of the mites, resulting in effects on metabolism and health. The intended level of poultry red mite infestation established will be such that the effects on birds will be largely limited to sub-clinical effects (i.e. not severe enough to cause observable symptoms), and more observable clinical effects of anaemia (i.e. reduction in red blood cells), weight loss and disease transfer associated with poultry red mite should not be seen in the chickens involved in our studies. Careful monitoring of the mite population in each group of birds will therefore be critical. The birds are likely to be exposed to mites for a total of 8 weeks to allow for the successful establishment of the mite population and subsequent administration of novel acaricides (i.e. substances that kills mites).

The initial studies aim to ensure that, for the majority of birds used in this project, the administration of novel acaricides (i.e. substances that kills mites) will not adversely effect on health or welfare of the chickens. The methods of handling birds and administering control substances have been chosen according to recommendations for poultry to minimise the amount of discomfort caused and limit this to the shortest duration 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)?

Protocol 1: Mild for >95% Domestic Chickens. The remaining birds will be sub-threshold.
Protocol 2: Moderate for <20% Domestic Chickens, and mild for the remainder.

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?

This investigation aims to quantify the protection provided to poultry against parasitism by poultry red mite following the administration of novel acaricides (i.e. a substance that kills mites). There are currently no reliable methods that do not use animals that can simulate the response of birds following administration of acaricides (i.e. a substance that kills mites) against poultry red mites. The novel products we will be testing have already undergone testing without using animals and have reached a point in product development that testing in chickens is required to test the effectiveness of products under commercially representative conditions to support bringing the product to market. Therefore, since we are aiming to test effective means of treating poultry red mite infestations in commercial hens, we cannot study this in isolated cells, or in other animal species. Commercial poultry red mite infestation and impact is complicated and ultimately can only be provided by, and experienced by a live animal. Although some of the cellular mechanisms can be studied in cells or isolated pieces of the nervous system, this would not enable us to determine effective product treatments for poultry red mite infestations to be used in commercial flocks. The use of live birds also allows for an evaluation of any negative impact on animal welfare under commercially representative conditions, which cannot be determined without using animals.

Hence the requirement to use live birds remains, in procedures that are considered to be relatively mild/moderate and not be dissimilar to the situation found on commercial UK farms where in some circumstances birds may endure a higher degree of predation by poultry red mite. In turn, the premises are often treated regularly for these mites (as well as other pests such as flies).

Which non-animal alternatives did you consider for use in this project?
No reliable laboratory methods have been identified which allow for commercially representative acaricide (i.e. a substance that kills mites) effectiveness testing without live animals being exposed. In previous work by this group, attempts were made to establish reliable methods that do not require live animals, in the form of a poultry red mite feeding system as an alternative to the artificial mite infestation on live birds.

Why were they not suitable?

In previous work by this group, attempts were made to establish reliable methods that do

not require live animals, in the form of a poultry red mite feeding system as an alternative to the artificial mite infestation on live birds. Whilst the results of this work were promising, mite death was high, and the repeatability of the method is currently extremely low. This is perhaps not surprising given the complexity of recreating the subtleties of mite-bird feeding in a test tube. Furthermore, any method utilising testing without live animals would not allow the safety of new acaricides (i.e. a substance that kills mites) to be tested.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 designed our studies carefully, using principles of good experimental design, so the minimum number of animals is needed to provide reliable data. The numbers of chickens have been carefully calculated using the appropriate statistical methods and data from previous related studies. In our initial studies, we have chosen to use 5 birds per treatment group to test the effectiveness of a novel acaricide (i.e. a substance that kills mites), while ensuring poultry red mite have access to a range of birds for feeding (i.e. to control against the potential unsuitability of an individual bird to host red mites). As we will be working with social species, this group size has additionally been selected to provide ample opportunity for birds to form social structures within groups, promoting normal and desired behaviours.

For our initial project, treatments will be arranged using principles of good experimental design consisting of five treatment groups, namely a negative control (water only), a positive control (commercial acaricide) and a novel acaricide product (at two concentrations), and the product formulation minus the active ingredient.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Careful thought has been given to both the proposed experimental design and the methodology necessary to realise the project aims whilst minimising the number of birds required. Power calculations (a statistical method to estimate number of animals needed) based on the observations from previous experiments have been carried out to determine the minimum number of birds required to observe meaningful effects for any work run under 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?

The number of birds required has been optimised through the use of laboratory screening methods that do not use animals to test substances beforehand (i.e. undertaken before any work would be done under this Licence), allowing treatment numbers to be minimised. The experimental design also involves pilot work (Protocol 1) to assure novel substance safety. Any substances that do not demonstrate safety at this stage will not be tested further, thus optimising the numbers of birds used at the testing stage involving the

exposure of live birds to the red mite.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Research to evaluate novel substances to control poultry red mite infestations in poultry requires the use of the species concerned. We have already conducted research in this area and so are using refined methods. Any products applied will be administered according to their final end-use in the most efficient and non-invasive means possible (e.g. by pressurised sprayer).

Under this Licence, birds will experience some discomfort during the administration of the novel experimental acaricides (i.e. a substance that kills mites), but no more than would be expected in a commercial setting during the application of such treatments. Exposure to poultry red mite in a controlled setting is also considered to cause only mild discomfort, since the level of poultry red mite used will be set at a normal (initially low) commercial level. Thus, even with mite population growth post-infestation, it is unlikely to result in the more advanced symptoms seen in severe commercial infestations where birds can die due to high levels of mite infestation. Based on our previous experience with poultry red mite, we do not expect rapid mite population growth to occur post- infestation sufficient to cause mite numbers to become excessive, thus creating an increased health risk to hens, e.g. anaemia (low red blood cell level in blood). However, since poultry red mite population growth can be very rapid, we will carefully monitor mite population development post-infestation through trapping, and then estimate the number of mites per bird over the duration of the study based on the trapped numbers. In order to ensure that birds do not experience excessive infestation levels, we will intervene to reduce mite populations if trapping data suggest that the mite population has exceeded 200,000 per bird, this being less than half the number of the mites present per bird in a heavy commercial infestation. Trapping will be undertaken at least weekly post-infestation to provide a regular assessment of the mite population(s). In the unlikely event that more severe symptoms are observed (e.g. clinical signs of anaemia and death resulting from extremely high levels of blood-feeding by mites) this would mean the termination of the study.

Birds will be housed in stable groups to promote the development of desirable social structures and behaviours. At times, the birds will be housed under conditions that reflect commercial farming practice. This is necessary to ensure our experimental conditions are as closely aligned to commercial practice as possible and where poultry red mite populations can establish under conditions that provide safe havens for mites and easy access to host birds.

Why can’t you use animals that are less sentient?

This investigation involves assessing the protection provided to laying hens against

parasitism by poultry red mites following exposure to acaricidal products (i.e. substances that kill mites). The novel experimental products we will be testing have undergone testing studies not using liver animals, so we are aiming to test effective means of treating poultry red mite infestations under conditions that are representative of those where these mites are a welfare issue, namely in adult hens in commercial egg- laying facilities. As a result, we cannot study other species of animal, and cannot work with younger birds (poultry red mite pose less of a problem at the rearing stage, since birds are reared typically in floor-based systems where there are fewer mite places for the mites to hide). As previously noted, poultry red mite infestation and impact is complicated and ultimately can only be provided by, and experienced by, a live bird. Choosing to do this study in simpler animal species would not enable us to determine effective products for poultry red mite infestations in commercial poultry.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Birds will be housed in stable groups to promote the development of desirable social structures and behaviors. Throughout the study, all birds will be handled by trained staff using low-stress methods (e.g. cradling) to minimise the impact of activities that require handling – e.g. data collection on bodyweight as part of animal welfare monitoring. Any novel acaricides (i.e. a substance that kills mites) applied will be administered according to their final end-use in the most efficient and non-invasive means possible (e.g. by pressurised sprayer).

Exposure to poultry red mite in a controlled setting is considered to cause only some discomfort since the level of poultry red mite used will be set at a normal (initially low) commercial level. Based on our previous experience with poultry red mite, we do not expect rapid mite population growth to occur post- infestation sufficient to cause mite numbers to become excessive, thus creating an increased health risk to hens, e.g. anemia (low red blood cell level in blood). However, since poultry red mite population growth can be very rapid, we will carefully monitor mite population development post-infestation through trapping, and then estimate the number of mites per bird over the duration of the study based on the trapped numbers. We will intervene to reduce mite populations if trapping data suggest that the mite population has exceeded 200,000 per bird, this being less than half the number of the mites present per bird in a heavy commercial infestation. Trapping will be undertaken at least weekly post-infestation to provide a regular assessment of the mite population(s).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The following best practice guidance will be used to ensure the experiments are conducted in the most refined way:

Effectiveness testing under Protocol 2: ECHA’s ‘Guidance on the Biocidal Products Regulation Volume II Efficacy - Assessment and Evaluation (Parts B+C)’ (Version 1.0 February 2017), with specific reference to Section 5.6.4.8 (pages 215-217) for testing on mites.

The housing of birds under Protocol 1: Code of Practice for the housing and care of animals bred, supplied or used for scientific purposes or Schedule 4 of the Welfare of Farmed Animals (England) Regulations (2007).

The housing of birds under Protocol 2: Schedule 4 of the Welfare of Farmed Animals (England) Regulations (2007).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project team assembled to work under this Licence are fully aware of the ethical and legal considerations relevant to the use of animals for scientific purposes and are actively implementing NC3Rs principles in this project. Importantly, our institution is an internationally-recognised university where adherence to the NC3Rs principles is a primary focus of all animal care staff and researchers. The project team routinely attends regular talks and discussions about animal welfare and the 3Rs through research presentations by staff and students at research meetings within our institution. Many of those meetings focus on developing new strategies to assess adverse impacts and promote positive welfare refinements in research animals, e.g. handling procedures to reduce distress. In addition, the applicant is the Chair of our institute's Animal Welfare and Ethics Review Body and highlights the importance of principles of the 3Rs extensively through research presentations (Refinement focused) and teaching activities he is engaged in within the UK and internationally. Therefore, the applicant is ideally placed to share advances in the 3Rs with the rest of the research team.

195. Cellular mechanisms controlling reactivity in small blood vessels

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cardiovascular disease, hypertension, arteries, endothelium

Animal types	Life stages
Rats	adult, juvenile
Mice	juvenile, adult, 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 isolate very small arteries from the microcirculation to study ex-vivo to allow us to discover how they operate to control local blood flow and blood pressure and how this changes in cardiovascular disease such as 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?

Understanding how the microcirculation works is key to unravelling what goes wrong in cardiovascular disease, which affects a great (>30%) proportion of the population in the UK and worldwide.

Dysfunction in the normal physiological working of the microcirculation precedes and predicts the appearance of atherosclerosis in large arteries.
What outputs do you think you will see at the end of this project?

Our research aims to discover how the diameter of the very smallest arteries in the body is regulated to distribute blood on demand to metabolising cells. By increasing resistance to flow, these vessels are the predominant influence on blood pressure. Understanding how the mechanisms operate normally and then deteriorate with disease will have major impact on the future direction of research by groups working in this area in the UK and around the world, and inform improvements in therapy. In the period of the project, we hope to identify the ion channels responsible for vasodilation and provide a mechanistic understanding of the coordination of microcirculatory function, showing how disease alters the signalling between cells that is necessary and sufficient physiologically.

Who or what will benefit from these outputs, and how?

All important discoveries will be communicated in non-specialist language to the general public, through University and department web-sites, as well as to the academic community through established means. Publication in the scientific literature is continual. Cardiovascular disease accounts for 25% of all deaths in the UK, with at least 30% of the population suffering from high blood pressure, so our research is topical, important and of widespread interest. Our fundamental research will provide new data to show how vascular disease causes dysfunction in the microcirculation, a change that occurs early on in the disease process, before more well defined structural changes are apparent in large arteries. A particular focus will be to show how loss of endothelial cell function links to the development of vasospasm in small coronary arteries, allowing us to identify novel therapeutic targets to oppose this unwanted response.

How will you look to maximise the outputs of this work?

Our discoveries will be communicated through peer-reviewed publications, including

reviews, and at national and international scientific meetings. In addition, we are regularly invited to deliver seminars and lectures at universities in the UK and overseas and we will use these events to disseminate our findings to interested and relevant audiences.

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.

Vascular changes with development and disease in both rats and mice are studied to understand the working of the human cardiovascular system in health and disease, enabling the design of new, more effective means to treat disease.

Typically, what will be done to an animal used in your project?

All animals on the project will be killed and arterial tissue taken for ex-vivo study. Mice will be bred with genetic alterations, which are not expected to cause harm. They will be killed and arterial tissue taken for ex-vivo study.

Rats with a genetic mutation will be used and the mutation is not expected to cause adverse effects during the time that the rats are alive in this project. Blood pressure measurements will be taken and then the animals will be killed after a maximum of 10 months (12 months of age) and arterial tissue taken for ex-vivo studies.

What are the expected impacts and/or adverse effects for the animals during your project?

None 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)?

GA mice are expected to suffer sub-threshold harms and rats are expected to suffer 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?

There are a number of reasons why this research is not possible without the use of animals. Mainly because vascular cells in culture change phenotype rapidly, so they are not representative of functional cells within the cardiovascular system. Another key consideration is that in life the artery wall functions physiologically as a syncytium, therefore to gain any meaningful understanding of vascular function it is essential we study intact blood vessels where smooth muscle and endothelial cells can interact. So there are no viable alternatives to the use of native vascular tissue.

Which non-animal alternatives did you consider for use in this project?

There are no feasible alternatives
Why were they not suitable?

N/A

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals requested is based on the variability of our previous experiments, enabling power analysis that signals the need to obtain replicates of at least 6-8 in each experimental series in order to define statistical significance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We always use as much tissue from each animal as we possibly can, and routinely carry out parallel experiments on different arteries from the same animal in order to minimize the number of animals required if this is possible. The majority of the animals we use are obtained from a commercial supplier, and as our protocols involve terminal anaesthesia there is no wastage 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?

Ex-vivo non-recovery experiments allow us to use as many arteries from a single animal as possible. We ensure we use arteries from different vascular beds from each animal, when possible to optimise 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.

We will use tissue from the rat and mouse. Rats represent probably the most widely characterized group of lower vertebrates as far as the regulation of the cardiovascular system is concerned. The majority of our ex-vivo data has also been obtained with this species. Vessels from the mouse will be studied to identify any notable differences and to form the basis for studies with genetically modified animals, as this species is amenable to genetic manipulation.

With regard to the use of spontaneously hypertensive rats, these develop raised blood pressure from around week 4 of age onwards, compared with WKY rat controls, mainly as a result of increased sympathetic nerve activity. Adverse effects have not been reported in these animals until they are over 12 months in age. Animals will not be kept alive for longer than 12 months of age, so none of the animals we use are expected to show any adverse clinical signs of hypertension or associated disease. Animals will be housed for 1-2 weeks, then the blood pressure measured by a non-invasive method in awake animals followed by schedule 1 kill. This will allow BP to be matched to age and weight of the animals, and allow us to quantify the extent of hypertension and relate this to experimental data obtained from isolated blood vessels. For the avoidence of doubt, as stated above AE are not expected to be seen in these rats, because they will not be kept alive beyond the age when clinical signs due to their genetic alterations would be expected.

Why can’t you use animals that are less sentient?

Because we need to use mammals that are warm blooded and have a vasculature similar humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All experiments will be be completed under anaesthesia, from which the animal will not be allowed to recover i.e. non-recovery. Commercially sourced animals are normally kept for the minimum time necessary to ensure recovery from the stress of transportation, typically 1-2 weeks.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice is adopted as appropriate when highlighted at regular user group meetings.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular electronic 3Rs newsletters relayed by the university

196. Development of new biological anticancer 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

oncolytic viruses, gene therapy, cell therapy, immunotherapy, cancer 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 develop new anticancer agents using therapeutic viruses and gene therapies. This includes developing viruses that kill cancer cells selectively and also using cells and viruses to express transgenes that can mediate an anticancer effect, for example through cancer immunotherapy or vaccination.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 around 367,000 new cancer cases in the UK every year (17 million worldwide) and around 165,000 deaths from the disease (9 million worldwide). Although treatments for certain types of cancer - such as leukaemia, lymphoma and melanoma - have improved in recent years, treatments for solid carcinomas (which represent the majority of the disease) have hardly changed. There remains a pressing need to develop new treatments for these solid tumours, most particularly when they are present at an advanced or metastatic state.
What outputs do you think you will see at the end of this project?

We will develop new cancer therapies based on emerging biological agents such as cancer-killing ‘oncolytic’ viruses that may be given by intravenous delivery to treat metastatic cancer.

As part of this, we will define factors and phenomena that limit the ability of these new agents to access and spread through solid tumour tissues. These might include components of the interstitial matrix (such as extracellular DNA for example) or elevated interstitial pressure decreasing fluid convection, and we would seek to identify strategies to reverse their effects. Current clinical trials show that inadequate spread through tumours can be a limiting challenge for many therapeutics

The outputs from these areas will manifest as scientific publications and lectures, and also as new anticancer agents for clinical development through early phase clinical trials.

Who or what will benefit from these outputs, and how?

The main beneficiaries will be patients with cancer, particularly disseminated cancer that is intractable to current treatment strategies. They will benefit from the development of improved new therapeutics. Two clinical trials are currently ongoing directly as a result of work we have performed in the previous 5 years, so we think this is an achievable goal. It will be supported by greater insight into tumour biology allowing improved design of therapeutic agents.

My institution and funding body may also benefit if the advances made in this project lead to successful commercialisation. Companies may also benefit if they develop these agents successfully.

How will you look to maximise the outputs of this work?

We are part of an extended international group of scientists who are developing new biologics based on genetically modified agents. Advances are discussed for peer scrutiny in both workshops and also in public meetings, following appropriate protection of intellectual property, and failed experimentation often leads to constructive discussion to explore and understand emerging biological principles. We publish all of our innovative findings as peer reviewed scientific publications in quality scientific journals.

Species and numbers of animals expected to be used

- Mice: We expect to use less than 4750 mice total in this project

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the animal species of lowest neurological sensitivity that can support the complex tumour architecture and microenvironment similar to human disease. We use only adult mice, which are more reflective of the majority of humans developing cancer.

Typically, what will be done to an animal used in your project?

Typical animal experience Brief inhalational anaesthesia, allowing painless subcutaneous injection of tumour cells. When subcutaneous tumour has reached 5-7mm diameter, single intravenous injection of therapeutic agent (using gentle restraint but not usually under anaesthetic). Restraint will be applied again for blood sampling from tail vein 4 times over 6h. Brief inhalational anaesthesia for intraperitoneal injection of harmless imaging agent after 24h with imaging (5 min) and then humanely killed by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals in this project will be implanted with subcutaneous tumours, but the majority of tumours are not expected to cause the animals any pain or distress. Tumour growth will be monitored very carefully and animals will be put down before any tumour-related toxicities arise.

The mice used in this project will experience several forms of discomfort. These include tumour growth, although care will be taken to prevent or at least minimise any suffering, radiotherapy of their tumours, immunotherapy, repeated anaesthetics, fasting for up to 24 hours, several injections and sometimes implantation of an imaging window into the skin above their tumours. We take great care to ensure that the suffering from each step is minimised, and individual animals receive only a subset of these interventions. In the majority of situations the most unpleasant procedure for them is likely to be repeated inhalational anaesthetic, which is very similar to that used clinically for 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)?

The majority of animals are expected to experience only relatively mild discomfort (for example with non-invasive growth of a tumour on their backs, followed by intravenous injection of therapeutic agents, monitoring of tumour size using calipers (without anaesthesia) and occasional anaesthesia to permit imaging (>85%)). The suffering of the animals receiving these treatments should be relatively mild, although the frequency of interventions means we have allocated this to severity band moderate. A small number of animals will receive more invasive treatment - for example they may receive radiotherapy as well as immunotherapy on their tumours, or they may be fasted for up to 32h (<10%). A few animals will have window chamber implanted so we can evaluate the tumour biology in greater detail using microscopy (<5%). These interventions are also graded moderate severity, although great care will be taken throughout to ensure that any animal which is suffering more than expected is humanely killed immediately.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals provide the complex biology we need to study in order to define factors limiting delivery of therapeutic agents through the bloodstream to reach cancers. This is important to enable us to develop treatments for advanced cancer that has spread around the body. *In vitro* cell systems do not allow us to study these factors.

Cancers grown in animals also provide useful tumour architecture, containing many different cells and cell types (not just cancer cells, but normal cells that are trying to 'heal' the tumour and thereby help it grow). This complex interaction of different cell types cannot be recapitulated using cells *in vitro* and hence we need to use cancer grown in animals.

Which non-animal alternatives did you consider for use in this project?

We make use of cells grown *in vitro* and also we make use of tumour biopsies from patients who are having their cancers resected. This is an excellent model of clinically-relevant disease, but sadly the tumour tissue survives for only a day or two once outside the patient and the amount of experimentation we can perform is limited. In addition the tissue is isolated and is not served by a functional blood supply, which limits its therapeutic relevance.

Why were they not suitable?

We use these models alongside animal work, but animal work provides the only system where there is a blood circulation (which is important to assess factors influencing delivery) and a functional immune system. In addition, only animals allow us to study anticancer efficacy because the tumour biopsies do not survive long enough in the laboratory for this 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?

It is important to ensure experiments are performed using sufficient animals that the results are clear and significant - to minimise the chance that they need to be repeated. To calculate the numbers of animals required we perform pilot studies to assess the variability of individual experiments and then calculate the number we need to use in the main experiment.

Typically we will have five researchers active on this project at any one time, and have estimated that each of them will make use of approximately 100-200 animals per year.

What steps did you take during the experimental design phase to reduce the

number of animals being used in this project?

The overall number of animals to be utilised will be minimized in several important ways:

Agents will be evaluated extensively in vitro prior to use in animals. This involves cell culture, computational and bioinformatic analysis.

In vivo experiments will use appropriate controls and animal numbers will be chosen to ensure that the data obtained are of statistical value. Control groups will be restricted as far as possible, and number of animals weighted accordingly. Designs will normally compare several agents and/or dose levels and factorial experimental design.

Use of pilot experiments: Optimising experimental models will be performed, using small numbers of experimental animals. These "optimisation" studies will be used to establish the best parameters for use in the experimental systems (eg. doses, timecourses), and will be based on our prior experience or on published methodology.

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 very important to allow us to choose the minimum number of animals needed for each experiment. We also make extensive use of in vitro models to try and minimise the numbers of animals we need to use.

We use the NC3Rs Experimental Design Assistant to help us make the experiment as efficient as possible, maximising the value of individual control groups and using design of experiments principle where we can.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 make extensive use of imaging approaches to maximise the amount of information we can obtain from each animal, without subjecting it to any invasive procedures. Imaging readouts provide an important technological advance, because individual animals can be imaged at different times, minimising variation due to the use of different animals at different times, and provide much better quality data (with far less variation) than could be achieved before such techniques were available.

Immunocompromised mice are also used to allow grafting of human tumours. This is important because many of our new therapeutic agents only work in human cells, and hence syngeneic murine tumours would not be very useful. These animals are kept in a sterile environment to protect them from pathogens, and the use of living tumours allows us to study the effects of physiological processes and complex tissue/tumour architecture on the activity of our therapeutic agents. These animals do not appear to suffer as a result

of their immunodeficiency.

Some immunocompromised animals will be administered human cells, to provide surrogate immune systems, and these animals may show signs of graft-versus host disease if the human cells react to the mouse. This can be minimised by only grafting cells from human donors that are known not to cause the problem, and that will be our approach. Animal suffering will be minimised by ensuring they are subjected to the minimum disturbance possible.

Some animals will be treated with radiotherapy because we are seeking to identify synergistic treatment combinations with our new biological and immunotherapy treatments. The treatment protocols are chosen to minimise unnecessary suffering to the animals, and stringent limits are enforced on the level of tissue damage that is allowed using the radiotherapy. This work is important because it may transfer directly to improved clinical treatments.

Fasting is a promising new way to improve cancer therapy, exploiting a difference in the ability of cancer cells and normal cells to defend themselves against nutrient deprivation. Several clinical trials are currently being undertaken. We will use limited fasting of animals (up to 24h) to see if we can augment the anticancer activity of our agents. Although humans might fast longer, the elevated metabolic rate of rodents means we will restrict the mouse fasting to 24h.

Some animals will receive window chambers placed above their tumours, so we can assess exactly what is going on in the tumour by microscopy. This provides a level of scientific detail and insight that is not available by any other means, and while it may be uncomfortable for the animals we take care to ensure that their pain and suffering is both minimal and tolerable so that it does not interfere with their normal behaviour.

Why can’t you use animals that are less sentient?

Mice are the animal species of lowest neurological sensitivity that can support the complex tumour architecture and microenvironment similar to human disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinement to tumour models: Most of our work will use subcutaneous transplantable carcinoma tumours, as our therapeutic viruses show broad spectrum activity against carcinomas of various types. We are particularly focused on colorectal and pancreatic carcinomas. Subcutaneous tumours provide simple cancer models, although they generally do not metastasise. Direct injection into subcutaneous tumours will use small volumes and long needle dwell times (to minimise back flow from the elevated interstitial hydrostatic pressure). Doses may be fractionated to allow delivery to different regions of individual tumour nodules, usually with just one access point through the skin.

Metastatic models may be achieved by injecting tumour cells intravenously (lung metastases). In each case animals will be assessed for 30 min immediately following administration of tumour cells and checked daily (and at least twice on the first day) to monitor for any toxicities or signs of pain, which will be treated eg. with wet mash, analgesics or euthanasia as advised by the NACWO/NVS.

For internal cancers (eg. pulmonary or intraperitoneal) pilot experiments using small numbers of animals will be used to allow characterisation of the kinetics and patterns of spread, to predict clinical signs and to define humane endpoints. Real time imaging using tumours expressing luciferase will be used where possible to allow assessment of internal tumour growth both in pilot studies and in definitive experiments. To allow assessment of occult tumour growth in experimental studies, control animals will be killed regularly throughout and inspected post mortem to assess tumour development.

Experiments using animals bearing lung tumours or solid internal tumours will be designed (on the basis of pilot studies or real time imaging) for termination at times expected to precede onset of significant disease pathology. Animals bearing fluid-forming internal tumours will be monitored visually for signs of fluid build up and palpitated daily. They will be humanely killed before the fluid causes any signs of distress and always before a waistline increase of 40% compared to age matched controls.

Some tumour cells will be genetically modified, so that following tumour growth they can be induced to secrete therapeutic agents locally. This will be useful in screening agents to inform which should be encoded within therapeutic viruses. No adverse effects are anticipated.

Human immune cells for adoptive transfer experiments will be acquired from suppliers where specific donors can be requested for repeat orders in order to build up a pool of ‘safe’ donors which minimise adverse effects and reduce the risk of any acute graft versus host disease.
Duration on study: Biodistribution studies using animals without tumours will generally be less than 7 days duration, although vaccination studies may last up to 100 days. Animals with tumours will be humanely killed when tumour or ill health endpoints indicate (typically 28 – 40 days) although animals showing tumour regression may be kept alive for up to 300 days to demonstrate long term benefits.

Light imaging: Non-invasive light imaging will be widely used to determine the activity of luminescent or fluorescent markers, either in the tumour cells or in the therapeutic agent. This relatively innocuous procedure can give good insights into growth of non-palpable tumours or metastases and will be very widely used for pilot studies of pulmonary or metastatic tumours.

General adverse effects and their control: Stress during administration will be minimised by careful handling of animals. Injectate volumes given will be minimised. With unexpected adverse effects the animal will be killed by a Schedule 1 method unless the effect is mild or can be adequately and promptly controlled by veterinary treatment or dose reduction.

Use of written protocols and score sheets: Written protocols are now used for all animal experiments, Score sheets will be used to monitor any experiments that show unexpected effects or toxicities

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the 3Rs principles as described on the 3Rs website (https://www.nc3rs.org.uk/the-3rs), and make use of the NC3R's Experimental Design Assistant in efficient design of experiments. All of our work also complies with the principle laid out in the Home Office Guidance on the Operation of the Animals (Scientific

Procedures)Act 1986

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The licence holder circulates 3Rs information, including the monthly newsletter, to all animal users. The recent advances in 3Rs practice are always discussed at the 3-monthly animal users meetings, which are compulsory for all animal workers.

197. Metabolic effects of sunlight

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with 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

Sunlight, Therapeutic benefits, Nitric oxide, Public-health, Metabolism

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 determine the impact of **ultraviolet A (UVA)** and the **visible spectra of sunlight in suppressing weight-gain** and related metabolic 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?

Many studies provide evidence that over-exposure to sunlight and repeated sunburns increase the risk of developing skin cancer. It is hence understandable that the government's advice regarding sun exposure is to avoid it. In recent years, this advice has been questioned, with recent research suggesting that low, non-burning sunlight may reduce the risk of developing a range of metabolic diseases such as diabetes, heart disease and liver dysfunction. This is extremely relevant and important as mortality rates from these conditions are up to 50 times greater than sunlight-induced skin cancers, on which advice is based.

Despite publications of the attractive health-benefits of sunlight, it is necessary to test these claims and accrue first-hand information and understand the actual magnitude of any effects, their limitations and potential side-effects. In order to do so, an animal model is necessary to verify the effects of light- induced suppression of weight gain and protection of downstream organs such as the liver that is often affected by excess fat and obesity. This is necessary before broadly similar studies are carried out with humans. These studies, regardless of the outcome (proving or refuting the hypothesis) are important evidence that form the basis upon which advice is placed.

What outputs do you think you will see at the end of this project?

The worldwide prevalence of obesity has nearly tripled between 1975 and 2020. Currently 39% of teenagers aged 18 years are overweight, and about 13% of the world's adult population are obese. Conversely melanoma - on which sun exposure guidance is based has declined, accounting for only 2 deaths per 100,000, and predicted to decrease even further.

The expected output from this project is the validation or rejection of the suggestion or claim that exposure to sunlight or certain wavelengths within sunlight can mitigate weight gain. The results of these investigations will be published.

This is extremely important in terms of public health policy and government guidelines, as should sunlight mitigate weight gain, it will allow governments, international bodies, and the global community to re-think policies currently set to 'avoid sunlight'. Should negative results instead be derived they are also equally valuable, as in this instance it will put to rest, claims made that sunlight is a potential 'elixir' against obesity, and related metabolic conditions.

Who or what will benefit from these outputs, and how?

In the short term (within the next 2 to 6 years) the project will provide new scientific information via peer- reviewed publications. This information will be of benefit to the

scientific community and also governments and other legislative bodies as it could potentially (depending on the results) alert them to the benefits of exposure to light, which is presently not factored in their advice to the public.

In the longer term (between 5 to 10 years) in the event that the results of these studies confirm the hypothesis, (that exposure to UVA and visible light can reduce the risk of metabolic disorders), the next stage would naturally be to progress into human clinical trials.

How will you look to maximise the outputs of this work?

We are ideally placed to publish this information and if necessary, affect policies thereafter. We have excellent internal policy groups, some specifically targeting diet and nutrition, others focused on sun exposure. These internal policy groups are useful as they feedback directly into policy making organisations such as local councils and the government. A prime example of this is the link between vitamin D (requiring exposure to sunlight) and COVID-19, as here an internal diet and nutrition group brought this directly to the government's attention via a written review. In addition to this, the work will be brought to public attention through scientific conferences and public engagement events.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Wild type (non genetically altered) Mouse strains between the ages of 4-20 weeks (not neonatal) have been chosen due to being used extensively for similar studies. Within these studies these mice, tolerate modified diet, light exposure and shaving well and derive results consistent with the overall aim. Adult mice strains have also been chosen as organs (for example the liver) evaluated after harvesting need to be fully developed.

Typically, what will be done to an animal used in your project?

For each strain processed:

Mice at 4 weeks of age will be fed a standard diet, but with no added vitamin D, herein referred to as low-fat diet.

All mice will stay on this diet for 4 weeks.

Mice will be ear clipped on one occasion and weighed as often as necessary, alongside body condition scoring and general observations for health status as listed in the protocol. This will allow us to establish whether light exposed, or topically treated animals have altered appetite in comparison to sham exposed.

At 7 weeks of age, an 8cm^2 area on the dorsal region of the mouse will be shaved. A

sedative or short general anaesthetic as directed by senior staff managing the animal facility (for example the NVS and NACWO)will be applied prior to shaving if required.

Between 8-20 weeks of age, 120 of the mice 80% will be switched onto a high-fat diet but with no added vitamin D, herein referred to as high-fat diet. The remaining mice will continue the low-fat diet.

At 8 weeks of age the mice will be separated into the following 5 groups: 'Baseline' controls:
Group 1. standard diet & sham light exposed
Group 2. High-fat diet & sham light exposed

(These groups will allow us to evaluate if the mice put on enough weight to visualise metabolic pathologies such as fat build up in the liver (termed liver stenosis), elevated glucose and insulin levels, and higher fat deposits elsewhere in the body, and serve as a negative control).

Light-exposed groups:

Group 3. Light exposed – high fat diet

(This group will allow us to evaluate whether UVA or visible light suppress weight gain and related metabolic disorders such as liver stenosis, or glucose/insulin levels)

Positive and negative controls

Group 4. treated with a compound termed ‘SNAP’, (a potent nitric oxide inducer. This constitutes a positive control group to assess if the potent signalling molecule nitric oxide (NO) is mediating any effect after light exposure).

Group 5 light exposed, and treated with a compound termed ‘CPTIO’ (that neutralises nitric oxide. This constitutes a negative control group to assess if the potent signalling molecule nitric oxide (NO) is mediating any effect after light exposure).

(To summarise groups 4 and 5 will allow us to ascertain whether the small signaling molecule nitric oxide, already associated with optimal function in other organs such as the heart mediates the effect of light on weight gain and other metabolic outcomes)

Mice in the groups above will undergo the stated treatments between 8-20 weeks of age twice weekly, at the same time on the same day to a shaved dorsal area of the mouse's skin.

At twenty weeks, the animals will be killed, and work assessing any related effects in addition to weight gain such as the severity of fat build up in the liver will be carried out. Mice will first be euthanised via a schedule 1 method, weighed and required organs frozen rapidly in liquid nitrogen. Liver weight, histopathology score, and circulating biomarkers will be used to ascertain liver function of treatment groups. Alongside the liver other organs will be taken, such as the heart, brain and kidney should these prove useful for further investigation or other related studies. Blood collected will be processed for measurement of additional biomarkers and potentially other relevant metabolites such salts that are released within the skin through light exposure.

What are the expected impacts and/or adverse effects for the animals during your project?

All animals on the high-fat diet may experience mild effects through consumption such as increased greasy coat, potential for dermatitis, reduced mobility around cage, and mild liver stenosis (fatty liver).

Mice will be weighed as often as necessary, with body condition scores also taken. All mice will be ear clipped so body condition and weights can be directly compared to each mouse held within the cage, Ear clipping, weighing and body condition scores will involve removal from their home cage. This may invoke stress, but this should be minimised by using experienced and trained staff, and acclimatising animals beforehand. We expect fewer than 5% of the mice to suffer from this.

At the end of the study all mice will undergo schedule 1 euthanasia, which will be carried out by trained and experienced staff so that the mice undergo as little pain and suffering as possible associated with this procedure. In a similar vein, a sedative may be administered prior to shaving if mice show signs of distress during the procedure. This will also be carried out by trained staff.

Topical treatments light exposures and shaving may invoke temporary reddening of the skin which is not expected to last for more than 24 hours. In rare instances. In cases of light treatment mice may present with oedema, blistering or cracked skin. This is expected to occur in fewer than 5% of the mice. We are confident of this as existing literature indicate that this has not occurred at all at these doses of treatment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not anticipate any of the mice to experience moderate severity, however it is possible that a small number of mice in groups exposed to UVA, drug treatments shaving or high fat diet may have these unlikely adverse reactions that reach this severity. In other studies data indicates this to be fewer than 5% of the animals. Hence the vast majority will be expected to experience at most, mild or sub- threshold 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?

A cell culture system in which we grow skin cells from a biopsy is currently used within our laboratory to evaluate the effects of sunlight within the skin. This has been beneficial for optimising which cells produce nitric oxide, the correct dose of light to apply in order to

achieve this and any adverse effects that occur. However, we have reached the limits that this or any other similar non-animal based alternative can provide. We cannot assess how different cell types, tissues and organs are affected by exposure of skin to light as there is no in vitro system that adequately recapitulate the systemic network of an animal. Also, we cannot assess if sunlight's effect on the skin would result in suppression of weight gain using non-animal models.

Which non-animal alternatives did you consider for use in this project?

There is no feasible alternative that could be realistically considered for the reasons described in the section above.

Why were they not suitable?

Less sentient living systems such as bacteria, fruit flies, and zebrafish, are not suitable due to the fact that the outer layer of their bodies are not similar to that of the human skin. As we are monitoring a skin- based effect and mechanism, we require an animal with a skin type that is more akin to humans to accrue the most reliable data. 3D culture system is also not suitable as our aim is to ascertain the effects of light on suppression of weight gain and systemic effects that impact on other organs such as the liver.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of mice to be used for this study is currently based on:

Previous similar work, in which pilot studies and projects have utilised on average 30 mice per group. This allowed clear and statistically significant result to be obtained.

Consultation with the Departmental statisticians, who agree that these numbers of mice would derive statistically significant results.

The proposed number also accounts for the possibility that we may need to use another mouse strain if positive effects are observed to ensure that these results are not strain-restricted.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Prior to designing the project a literature search was carried out. Six publications were found and assessed in detail with conditions, and other specifics of the experimental set-up collated as reported.
A very detailed pilot study reported on the numbers of mice necessary for obtaining statistically significant results. The study suggested 25-30 mice per group.
Supplementary material from each study, and email communication with the research

teams that carried out the pilot study allowed validation of the numbers while taking into account any potential adverse effects they experienced, as well as the frequency of such effects.

I reached out to experienced members of the Department for guidance and discussions. senior departmental statisticians who confirm the suitability of the number of mice, and continue to work with us ensuring the number remains statistically significant, whilst identifying opportunity to reduce this to the lowest possible number.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Firstly, we will be buying the exact numbers of mice required. Secondly, staggering mice, ensuring only a small subset are initially subjected to drug, diet and light exposure (shown universally to work in other studies with minimal side effects) allows us to test documented literature first hand. Thirdly, consulting with departmental statisticians once this small set of animals have been processed, deriving power calculations based on data obtained, allows us to verify if the n-number can in fact be reduced while retaining the ability to obtain statistically significant results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Extensive reading prior to planning the project suggests the mouse models we intend to usehave been used in this research area extensively over the past ten years. The known effects relating to high fat diet light-based treatment and topical drugs on mouse physiology are well established.

The use of UVA and visible light, the more benign wavelengths of sunlight have been chosen when planning out the project over the short wave UVB radiation due to being at least 10,000-fold less damaging to the skin.

Mice are to be used at a relatively young age, and will as such be of optimal health, not displaying potential problems that could occur if we age the mice further-such as obesity through non-modified diet.

The reduction in procedures such as taking blood and serum for assessment of immune functionwhile alive have been replaced by post-mortem techniques. This ensures procedures carried out are refined to the lowest possible level.

Topical treatments reported not to be toxic, and invoking only a local effect have been opted for over ingested, injected alternatives that could have a systemic effect.

All animals will be provided with comfortable group housing and enrichment. We do not expect any adverse effects but analgesics and or sedatives and increased monitoring will be used if considered appropriate.

In the case of shaving the skin a brief general anaesthetic may be used if required to make the procedure as stress free as possible.

Why can't you use animals that are less sentient?

Bacteria, fruit flies, and zebrafish, are not suitable due to the fact we are monitoring a skin-based mechanism and require an animal with a similar skin type to humans to provide the most reliable data.

Mice under terminal anaesthesia or at an immature life-stage cannot be used as this is a study being performed over a prolonged timeframe of 20 weeks hence we need an conscious mouse. Furthermore, we also need the mice to gain weight through a high fat diet and need to assess this effect on fully developed organs and serum at the end of the experiment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In vitro based techniques have been and will continue to be used to optimise lab-based protocols prior to use on animals. This reduces animals that would otherwise be used for this process. Cadavers will be used to practice procedures ensuring the procedure is carried out accurately and confidently in the quickest possible time, keeping the level of stress the animal is exposed to, to a minimum. An appropriate monitoring strategy successfully applied on other papers published will be used here to monitor mice after exposure to UV light, looking at potential adverse effects such as oedema, ensuring health concerns are picked up as early as possible.

While we would wish to measure the effect of other parameters such as altered blood glucose and insulin resistance via sunlight with blood clearance tests over time, these procedures would cause prolonged unnecessary distress. As such, we have opted to obtain data by carrying out as much assessment of these parameters after schedule 1 euthanasia, via harvesting serum and organs and carrying out wet bench work. This reduces pain suffering and distress to procedures to a minimum. prolonged up-regulation of genes and biomarkers within the blood after euthanasia has allowed us to do this.Extensive review of previously performed experiments will allow us to use established well proven procedures. This minimises harm to the mice associated with testing new parameters. In addition to this, we will expose the mice to light and drugs in a staggered manner so that any unexpected adverse effects are experienced by the minimal number of animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

THE NC3R's, NICE, ARRIVE, FELASA and Home office guidelines such as the guidelines laid out in the project and personal licence will be followed in order to carry out best practice.

How will you stay informed about advances in the 3Rs, and implement these

advances effectively, during the project?

During this project, we will regularly follow the FELASA, NC3R, LASA websites and newsletters and also review the literature for novel methods and techniques to improve our project in accordance with the 3Rs. We also aim to participate in regional NC3R's seminars and updates when possible and if relevant to LASA/NC3R's meetings or meetings recommended by LASA/NC3R. We will also follow announcements from companies within the field who are developing a human cell line model pertaining to specific disorders, that can be implemented as a skin cell model with the potential aim to replace and reduce the number of animals when possible.

198. Identifying molecular targets for malaria intervention

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

malaria, parasitology, transmission, mosquito

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 increase our fundamental understanding of the molecular mechanisms that underlie malaria parasite development in the vertebrate host and insect vector, and to identify new parasite molecules and molecular pathways to target parasite 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?

Malaria remains a serious global public health problem affecting millions and killing over 400,000 people annually, mainly in the under-fives. Malaria control strategies are hampered by increasing parasite resistance in the field to established antimalarial drugs, as well as by widespread insecticide resistance in its mosquito vectors. There is therefore a great need to develop additional and novel strategies to prevent and treat parasite infection. These include novel parasite-directed ways to control malaria transmission by mosquitoes.

What outputs do you think you will see at the end of this project?

This project will contribute to our general understanding of malaria parasite biology and will identify new targets and intervention strategies for parasite control. This information will be presented in the form of peer-reviewed publications in Open Access and ARRIVE-compliant scientific journals where possible. Related metadata will be made available in appropriate, publicly accessible repositories.

Who or what will benefit from these outputs, and how?

Our outputs will inform the scientific community and can then be taken further in human malaria studies or studies of related parasites to ultimately lead to the development of new antiparasitic chemotherapy or vaccines, which will have great potential benefit for global public (and animal) health.

Immediate benefits (during lifetime of project licence): identify new targets and intervention strategies for parasite control in the mouse model. Potential beneficiaries are other researchers in the fields of malaria and related parasites.

Mid-term benefits (5-10years): validate new targets in the human malaria context, which can be taken forward for development of chemotherapeutic drugs. Potential beneficiaries are the research community and pharmaceutical industry.

Long-term benefits (10-20years): Application of new antimalarial measures, as well as other antiparasitic drugs against related parasites (e.g. Toxoplasma, Cryptosporidium, Neospora, Babesia). Potential beneficiaries are human and veterinary patients, the Health sector, and wider society.

How will you look to maximise the outputs of this work?

We will maximise our output by collaborating with experts and timely dissemination of new data through scientific publication, presentations at meetings/conferences, and press releases.

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 using adult mice as they are necessary to grow and maintain rodent malaria parasites.
Typically, what will be done to an animal used in your project?

Procedures include infection with malaria parasites by injection or by infected mosquito bite; repeated monitoring of parasite levels in the blood; harvesting parasite material by collecting the blood; chemotherapy treatment by injection or oral administration. Typical experiments have a duration of 1-2 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Mosquito feeding can cause short-lived irritation at bite sites (<24h). Infection with rodent malaria parasites can induce moderate symptoms including piloerection and lethargy (<24h).

Expected severity categories and the 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 of the animal experiments is moderate. However, in the vast majority (>99%) of animals the scientific endpoints will be reached before the moderate severity limit. Thus, most animals are expected to experience only mild discomfort or symptoms. Any mice reaching moderate severity will be humanely killed.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 encompassed the entire *Plasmodium* life cycle with a focus on its development in the mosquito vector. Rodent malaria models have been at the forefront of experimental research of *Plasmodium* development in the insect. Laboratory infection of mosquitoes with rodent malaria is relatively easy. Furthermore, rodent malaria parasites are amenable to genetic manipulation, which has served as a key tool for functional studies. Rodent malaria is also an excellent model for studying parasite development in the vertebrate host, and for studying parasite transmission from host to mosquito and mosquito to host.

Which non-animal alternatives did you consider for use in this project?

In vitro culture of blood stage rodent malaria parasites in the presence of rodent red blood cells.

Why were they not suitable?

In vitro culture of rodent malaria parasites in the presence of suitable host cells is not suitable for sustained propagation of the parasite. Thus, parasite culture does not provide a viable alternative to the use of live animals for parasite maintenance or production, nor for studying parasite infection in the vertebrate host.

The mouse is the most appropriate and highly defined host for rodent malaria models. Rodent malaria species such as *P. berghei*, *P. chabaudi* and *P. yoelii* are widely used and suitable animal models of malaria. They are relatively cheap, versatile and safe.

Genetically, the mouse is a highly defined host with a complete annotated genome, and many strains (including genetically altered) are available.
Experience has shown that many aspects of malaria biology, including transmission, are shared between mouse and human parasite species. The mouse malaria model is thus a very useful tool for basic parasite biology studies and the identification of potential molecular targets for intervention.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These 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 licence is for a continuation of a longstanding research programme spanning over two decades. This extensive experience informs our estimation of animal usage, whilst ensuring that individual experiments use minimal numbers of animals to answer our scientific questions and test our hypotheses. The number of mice required is based mostly on animals needed for parasite maintenance and production.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments that require statistical analysis are designed to use animal numbers that give appropriate statistical power to show the required differences in the context of experimental variation. Power calculations are performed using NC3Rs Experimental Design Assistant and reviewed by the AWERB statistical advisor at our institution. Generally, experiments are repeated twice to ensure reproducibility of results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In some cases it is appropriate to present parasitized blood to mosquitoes in membrane feeders, for instance to test the effects (ex vivo) of the presence of potential transmission-blocking drugs or antibodies on transmission. Whilst this does not replace animal use *per se*, it does reduce the number of procedures carried out on animals. Generally, however, direct feeds are vastly superior over membrane feeds in establishing mosquito infection with malaria parasites and is therefore the method of choice as poor/unsuccessful mosquito infections ultimately lead to more animals being needed.

Pilot studies involving the smallest number of animals possible will be conducted when new hypotheses are tested. We are constantly optimising our procedures for biochemical analyses of parasites so they can be carried out with smaller amounts of starting material, which results in using fewer animals for the production of parasite material.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 mouse model in combination with rodent malaria parasite species. Procedures include infection of adult animals with malaria parasites by injection or mosquito bite; monitoring of parasite infection level in the blood by microsampling; harvesting parasites by blood collection; and chemotherapy treatment by injection or oral administration.

The vast majority of animals are not expected to proceed to the moderate severity level and only mild (or no) clinical symptoms are expected to develop before the scientific endpoints are reached. The reason for the moderate severity limit is that in some animals infections may develop more quickly than anticipated, and in those cases symptoms may reach moderate severity level (in communication with NACWO). Based on experience, this is anticipated to happen in less than 1% of animals.

Why can’t you use animals that are less sentient?

Plasmodium species require the vertebrate host and mosquito vector to complete the life cycle. Animals are infected to allow parasite multiplication for assessment of parasite development, parasite production, or transmission to mosquitoes by blood feeding. Adult animals are most appropriate for these applications.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all animal experiments, procedures will be carried out following local best practice guidelines based on the latest LASA guidelines. These include guidelines for dosing and sampling regimes based on published guidelines.
We will administer chemotherapeutics that have established safety and efficacy profiles and that are known not to cause more than transient mild effects in mice.

Mosquito feeding on mice is required for several reasons: (1) parasite lines lose their ability to be vector transmitted after serial blood passages; it is therefore imperative that occasionally the parasite is transmitted to and from mosquitoes via direct feeding on an animal. (2) to assess the ability of transgenic or treated parasites to be naturally transmitted. All mosquito feeding is carried out under general anaesthesia. Mosquito feeding may cause short-lived irritation at more tender sites of the body (snout, mouth, vulva, anus, hands, feet). To protect sensitive areas, they will be placed beyond the mosquito-accessible area, or be protected with micropore tape. We will also use analgesia after mosquito feeding to reduce discomfort. Based on average bloodmeal size (1.5 microliter), the risk of anaemia will be limited by using no more than the stated number of mosquitoes. In the case where naive mosquitoes are infected, the animal is humanely killed after feeding before it wakes up.

A clinical scale of symptoms is in place to identify humane endpoints. We will pilot the use

of the grimace scale as a potential refinement.

To minimise the effects of parasite infection on the animals, infection times are kept to a minimum, while ensuring that the scientific targets can be reached.

Genetically altered parasite lines are not expected to give rise to more severe clinical disease than parental lines.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office, NC3Rs (e.g. on microsampling) and LASA guidance (e.g. on substance administration), disseminated to us through regular email updates from our Establishment.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will read NC3Rs guidance/newsletters disseminated to us through regular emails to PPLHs and PILHs from our Establishment.

199. Genes affecting developmental and degenerative diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Stem Cells, Vision, Central Nervous System, Developmental Biology, Degenerative Disease

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the etiology of genetic diseases affecting human eyes by modelling and studying them in mice. New potential therapies that can potentially significantly improve the quality of life for patients suffering from genetic diseases of the eye will be trialled in animal models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Around 3% of babies are born with genetic abnormalities that will significantly affect the quality of their lives. Understanding genetic disease is fundamental to improving outcomes for patients, by understanding the processes of development or tissue maintenance that are abnormal, and therefore to predict drugs or gene therapies that may help affected people. We focus on genetic disease and abnormalities of the eye. Problems with eye growth during embryogenesis contribute to the very high levels of refractive error (short- and long-sightedness and astigmatism) seen in modern society, and can lead to blindness. Degenerative corneal disease is the leading cause of blindness in developing countries, and to tackle this problem it is necessary to understand the developmental biology of the eye, its maintenance and wound healing responses, and importantly the specification and function of stem cells that maintain the eye. The objective of this project is to understand the genetic and stem cell basis of human genetic diseases and to trial new drugs. This is essential for formulating medical strategies for next stages of treating human disease.

What outputs do you think you will see at the end of this project?

Assay of at least 2 drugs with potential to improve the ocular surface in mice with eye degeneration such as aniridia-related keratitis. This will be measured through publications of efficacy and investigation of mechanism, and if successful will lead to further translational trials to bring a functional therapeutic to medicine.

Understanding of the origin and specification of stem cells maintaining the retina and ocular surface and modulating ocular wounding.

Scientific knowledge of genes underlying eye abnormalities including microphthalmia (including refractive errors such as myopia) and glaucoma.

Outputs will include:

Scientific publications in peer-reviewed journals.

Dissemination to patient groups and popular articles.

(Hopefully) validated drugs that can be taken further to human clinical trial.

Who or what will benefit from these outputs, and how?

The immediate benefit (0-3 years) will be to the scientific community, especially others working in stem cell, developmental and ophthalmological fields. The project will answer important outstanding questions that will inform future directions for research projects.

Longer term (3-5 years), the identification of genes affecting eye development will allow and inform screening for previously unidentified myopia and glaucoma genes, among other disorders.

It is also the intention that patients, e.g. aniridia groups, will benefit from the translation of therapeutic drugs (5-6 years) to ameliorate or reverse currently untreatable aspect of eye disease including microphthalmia and aniridic keratitis.

How will you look to maximise the outputs of this work?
Collaboration with other groups working on different but overlapping fields to disseminate knowledge and share experiences of mutations and successful/unsuccessful experimental

techniques.

Liaison with patient groups and presentations to clinical and preclinical workers.

Dissemination in popular formats such as in 'The Ophthalmologist' and other professional outlets.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are chosen as a model mammalian species with multiple inbred, genetically modified lines available. As mammals, mice are genetically closer to, and more representative of, human, for most aspects of their biology, than other potential vertebrate models such as chickens, fish, frogs. Because the genetic background of the mice is controlled, experiments are replicable, without population or individual genetic variation. The availability of multiple pre-existing strains reduces animal usage required to produce new strains. Embryos and fetuses are used to study the roles of genes during embryology ands stem cell specification. Adult mice are necessary for understanding maintenance and disease in adult humans (when there is a balance between tissue damage and repair that needs to be maintained). As we have previously shown that stem cell activity may decline with age, and for the study of degenerative disease, a number of aged mice (up to 2 years old) may be maintained.

Typically, what will be done to an animal used in your project?

The typical experience for a mouse used in this project is an experiment lasting from a few days to several weeks, with one or two procedures and only transient discomfort e.g. from injections.

Mice may be born with a Mild genetic defect that does not cause distress or pain but may typically e.g. cause poor vision. They may be mated with other mice carrying genetic modifications. Most mice will be used without further procedure, but substances will sometimes be administered that either label cells for visualisation (e.g. iododeoxyuridine, given in drinking water for a period of three months or by injection for up to two days, with no toxicity), that turn genes on or off (e.g. tamoxifen, by one or two subcutaneous injections), or that are potential therapeutics (in drinking water for 3 weeks, or by use of eyedrops over a similar period). No mouse will undergo continuous administration of drugs for its entire life, but follow-up may require mice to be monitored for several weeks or months after treatment. A small number of mice may undergo minor procedures under anaesthetic (such as a small epithelial wound) with recovery to assay wound healing in disease and therapy. If a mouse were showing significant deviation from normal health, it would be humanely euthanised under veterinary advice.

What are the expected impacts and/or adverse effects for the animals during your

project?

Mice may experience very short-lived discomfort due to injections or administration of harmless substances orally. Short-lived (24 hour) weight loss is sometimes observed after administration of tamoxifen, but mice quickly recover normal weight. Mice experience uncoordination when recovering from anaesthetic, and may experience mild irritation during periods of wound healing, which rarely last longer than 24 hours. Very occasionally, mice may fail to recover from anaesthesia, or experience postoperative weight loss (>10% of body weight, not recovered in 72 hours) and would be humanely killed.

Mice may experience a phenotype as a result of genetic modifications they carry. in some cases, the phenotype is well established and predictable, e.g. Pax6 mutation causes eye defects that are lifelong, lower visual acuity but do not affect the ability of the mice to interact, feed and breed. Other phenotypes may not be predictable, if mice are mosaic for a harmful mutation in genes such as Sox9 or other developmental/stem cell genes, however levels of mosaicism are controlled to avoid pain, distress or inconvenience to the animals. Any animal experiencing negative health due to uncontrolled, unexpected genetic defect will normally be humanely euthanised on veterinary advice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 25% of our animals will exhibit eye defects that affect visual acuity. Mild.

Approximately 2% of our animals will undergo an operation under general anaesthetic with recovery and healing. Mild.

Approximately 50% of or animals will undergo administration of substances (may overlap with those above). Mild.

Approximately 50% of animals will carry genetic modifications that are subthreshold, but overlap with classes above i.e. they will experience substance administration of Mild severity.
Induction and maintenance of general or local anaesthesia, sedation or analgesia to mitigate the pain, suffering or distress associated with the performance of other regulated procedures will be indicated by using the following codes in protocols: AA (no anaesthesia); AB (general anaesthesia with recovery); AB-L (local anaesthesia); or AC (non-recovery general anaesthesia).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to investigate lifelong influences on stem cell biology and tissue health in complex three dimensional, multi-tissue systems that cannot be fully recapitulated outside the animal, or for sufficiently long periods of time.

Which non-animal alternatives did you consider for use in this project?

In vitro cell cultures using human cell lines.

in silico modelling of cell migration in organ systems.

Maintenance of genetically modified induced pluripotent stem cells derived from genetically modified animal cells.

Why were they not suitable?

Methods above are effective at replacing animal experiments and ultimately reducing the number of animals we need to use. We continue to develop retinal and neural (brain) organoids that will enable us to further replace animals, however none of these methods yet have the level of organisation, sophistication and multi-tissue contribution (e.g. vasculature) that fully recapitulate the in vivo situation. For investigation of complex organ systems, especially in this case for assay efficacy and safety of new potential therapeutics, we can reduce the number of animals using in vitro techniques and computer modelling, but validation of results obtained ex vivo still required use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?
Estimates of numbers of animals are based on both a prediction of the number of animals we require for the strands of the programme of work, and the numbers of breeding stock that must be maintained. This requires an assessment of optimisation and efficiency of breeding regimes. Estimates are also based on previous experience of mouse usage for previous projects on which the current proposal is based.

For example, to administer two potential therapeutic drugs per year through two different modes of administration, given the need only to show potential therapeutic effects, it is estimated above this will require 48 mutant and 48 control mice, which with associated breeding is likely to require maintenance of at least two breeding pairs. Adding wound healing onto this (maximum 20 mice) and associated labelling experiments and analysis predicts usage of 150 mice per year for that aspect of the project.

Triple GM (e.g. Cre-positive, LacZ reporter positive, flox allele) mice inevitably have larger numbers in breeding regimes in that they produce multiple essential controls for each 'experimental' mouse, and given the estimated usage of 100 experimental mice per year for stem cell function (40 adult mice) and specification (60 fetal mice) on a more complicated

breeding regime with multiple genotypes where only 25% of the progeny from breeding are Cre+ Reporter+ flox/flox, this will require breeding of 160 mice per year (for postnatal work) and another 30 timed matings (for fetal work) requiring total 220 mice per year per gene/strain.

The stem cell/ eye size/microphthalmia gene expression studies are continuations of ongoing studies that (based on previous mouse returns) are using 100 mice per year for breeding and analysis. With no expansion of mouse usage foreseen, we therefore predict 470-500 mice per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have massively reduced wastage in mouse breeding regimes by moving, when approriate to a [Cre-negative, Flox/Flox, Reporter-positive] x [Cre/+, Flox/+, Reporter-positive] mating regime which can produce 25% experimental Cre+ Flox/Flox and the various controls, over previous breeding regimes where only 1 in 16 mice was Cre+ Flox/Flox.

Fundamental to experimental design based strategies for reduction in number of animals is robust molecular and histological analysis techniques to ensure that no mice are wasted through technical failures and having to repeat. We test all reagents and techniques in vitro before incorporating them into in vivo experimental design, to minimise wastage and prevent duplication of animal procedures.

Experimental procedures are designed in accordance with NC3Rs guidelines to ensure unbiased, powerful studies that are amenable to robust statistical analysis, as described at https://nc3rs.org.uk/experimental-designstatistics

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 has been described above. Pilot studies are routinely performed when new treatments or experimental protocols are being trialled to 1) ensure no unexpected adverse effect and 2). As described above, we are using predictive modelling to assess the likely magnitude effects associated with treatments that affect parameters of cell migration or adhesion. This allows us to estimate the power of the experiments and adjust animal numbers accordingly.

When possible, we consider whether animals used for one study can contribute to other projects within the lab or with collaborators. For example studies where DNA labelling has been performed to study stem cells in the eye are routinely shared with colleagues studying other tissue or organs systems.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are used as a mammalian species with eyes and CNS that mirror human, with high conservation of gene sequence, expression and function. Genetic mutations used are subthreshold or mild, and we reduce potential distress or suffering to mice carrying potentially more severe mutations by use of mosaics that carry only a minority of mutant cells in an otherwise wild-type environment. The use of drinking water to administer substances whenever possible significantly refines the experiments and avoids repeated injections or use ofminipumps.

Why can't you use animals that are less sentient?

We need the animals to have vertebrate camera eyes, and this precludes use of Drosophila or other invertebrates for experiments included in this Project. We do however use less sentient and immature models - for example chicken embryos are fundamental to our studies of eye development and these reduce the number of protected animals that must be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Routes of administration of substances will be non-invasive when scientifically possible, including via drinking water or eye drops. Animals will be trained when necessary to acclimatise to eye drops when possible.

We have optimised a method of tamoxifen administration requiring only 1 injection rather than 2-3 and which does not make mice sick. We will continue to reduce tamoxifen administration to minimise recombination events in mosaic mutant animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Mostly we follow NC3Rs guidance with specific relevance to UK animal experimentation, such as best practice guidelines published by Prescott and Lidster (2017). Other NC3Rs material available at http://www.nc3rs.org.uk/3Rs-resources will be used in combination with local expertise. The ARRIVE Guidelines 2.0 are also followed to receive, disseminate and inform best practice (Percie du Sert et al., 2020)

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.1217https://www.nature.com/articles/laban.1217.pdf

Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLOS Biology 18(7): e3000410. https://doi.org/10.1371/journal.pbio.3000410

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of the NC3Rs Project Grant Funding Panel, and receive all relevant

publications and action plans ongoing in NC3Rs.

Home Office

200. Targeted treatment of blood-borne 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

Blood filtration, Sepsis, COVID-19, Extracorporeal, Anti-inflammatory

Animal types	Life stages
Pigs	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 aim is to assess the safety and feasibility of a novel medical device, a Magnetic Blood Filtration System, which is used in an extracorporeal system (outside the body) to remove harmful pathogens from the patient's blood stream.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 Blood Filtration has the potential to provide targeted and fast treatments for blood-borne diseases. The magnetic blood filtration technology enables the selective removal of harmful components, such as cells, bacteria, toxins and inflammatory cytokines

directly from a patient's bloodstream. The ability to precisely extract unwanted disease causing substances in this way has the potential to revolutionise the treatment of deadly blood-borne diseases, such as sepsis and COVID-19, leukaemia and malaria. Regulatory animal studies are required prior to human clinical trials.

What outputs do you think you will see at the end of this project?

The expectation is to validate this novel treatment, (the Magnetic Blood Filtration system), for blood- borne diseases, including COVID-19 and sepsis. This will include data for regulatory submission prior to entering human clinical trials. In addition to safety studies for regulatory submission, efficacy studies will also be performed which will lead to advancement of knowledge in treating these diseases and publication of this.

Who or what will benefit from these outputs, and how?

The ability to safely and effectively remove blood-borne pathogens via a targeted approach has huge potential to treat a number of serious medical conditions and diseases. These range from inflammatory diseases such sepsis and COVID-19, to blood-borne cancers, to malaria. Work using this filtration system for Malaria is already in human clinical trials.

In the short term this work will focus on COVID-19 and sepsis. COVID causes an intense 'hyperimmune' response and studies have shown that over 50% of hospitalised COVID patients develop sepsis. Sepsis is caused by pathogens from an infection which circulate in a patient’s bloodstream. COVID and sepsis can both elicit a abnormal immune response that can escalate to multiple organ failure and death. The challenge in sepsis is that both the pathogens and the body’s own response contribute to the disease. The ability to immediately and effectively treat and control the inflammatory response could significantly improve mortality and morbidity in these patients.

In particular, there is an urgent need for new therapeutic tools to help clinicians reduce the severity and mortality of COVID-19 and the number of patients that end up on ventilators. Clinical studies have shown that much of the harm done in severe cases of COVID-19 results from the body's overactive or hyper-immune response. Using the magnetic blood filtration system (MBF) to reduce this hyper- inflammatory state without systemic delivery of biologics, provides an early opportunity to control the immune response without causing long-term immunosuppression. MBF could treat both the hyperinflammation seen in the lungs that drives hospitalisation and the secondary sepsis in COVID-19 patients that results in significantmortality.

How will you look to maximise the outputs of this work?

The outputs from this work will be disseminated to the wider scientific community through publication in peer reviewed journals and by presentation at international meetings. Negative data will also be published and shared within the scientific community. Where appropriate, patients and the public will be informed of the outcomes through appropriate avenues.

Species and numbers of animals expected to be used

- Pigs: 40
- Sheep: 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.

Adult animals are ideal for this study for fulfilling scientific objectives, based on anatomy, size and expertise of scientists and animal care staff. Sheep and / or pigs may be used for these studies as suitable models for both the safety and efficacy work, based on our already gained scientific knowledge working with both these species in this specific scientific field.

Typically, what will be done to an animal used in your project?

Animals will be acclimatised and trained for handling purposes.

In the first study, animals will be anaesthetised and connected to the Magnetic Blood Filtration System, before being terminated (first group) or recovered (second group). Animals in the recovery group will be recovered for up to 1 month following the procedure and serial blood tests could be performed for the assessment of the longer term effects. Analgesia and antibiotic treatments will be provided post recovery and animals will be monitored by clinical observations. Animals will be killed and post mortem assessments performed.

In a second terminal study, animals will be anaesthetised and efficacy testing performed by administering compounds designed to elicit an inflammatory response (e.g. LPS) whilst connected to the Magnetic Blood Filtration System designed to remove or reduced inflammatory factors released.

What are the expected impacts and/or adverse effects for the animals during your project?

The impact of any adverse effects expected for animals receiving compounds to elicit an inflammatory responses are expected to be minimal as this is performed under terminal anaesthesia.

Previous animal studies conducted in sheep have shown use of the magnetic blood filtration system has minimal adverse effects, however, there is always a risk of thrombosis of the jugular vein, which would pose a minimal overall risk to the animal but which could lead to local swelling. There is a small risk of thrombophlebitis (infection of the veins) in the recovery period, which could potentially lead to systemic infection; this risk will be mitigated by antibiotic administration and jugular vein access only being used briefly and in a clean surgery room.
In the recovery studies the procedure itself and the recovery are anticipated to be associated with very little discomfort. If there is any short-term mild pain this will be controlled with appropriate treatment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For recovery studies, moderate in 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?

Pre-clinical safety studies conducted in animals are required as part of the performance validation of a medical device. International standards (ISO10993 part 4) detail the requirements for biological evaluation of medical devices and list the selection of tests required for the pre-clinical testing of devices interacting with blood. These tests require the use of animal models.

For efficacy studies only a whole biology systems approach will give conclusive evidence of treatment of the hyper-inflammatory conditions the device is designed to treat.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to achieve the aims of this project for the reasons stated above.

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 first pilot studies will likely use 1-4 animals to validate the set-up and management of the magnetic filtration system and are designed to test at an early stage any obvious correction of the protocol that would be required. Pilot studies will also inform the status of physiological status of the animals prior to embarking on recovery studies.

Recovery studies will be designed to satisfy regulatory requirements using the minimum number of animals.

Finally terminal studies designed to test efficacy will be conducted . Work involving administration of the inflammatory compounds in large animal models is already established as a known working model at the test facility, as well as bench top testing of blood and a known ability to measure outcomes in the blood; these together will reduce numbers of animals as some optimisation has already been done.
The information from these previous studies has been used to inform the number of animals required for this project application.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The present studies are designed to provide data on safety and efficacy of the device, as such, there is no formal calculation of number required that is possible.

The number of animal appropriate to satisfy minimum MHRA regulatory requirements for the safety studies will be used.

In the efficacy studies, optimisation of the inflammatory large animal model has already been performed which will reduce animal numbers. Regular review of data after each study will optimise the use of data and

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Training with the MF system will be conducted at the test facility with blood from a cadaver to familiarise the test facility team with the system prior to use in animals.

We will use data/ experience from previous research performed at the test facility to provide the initial validation data for the efficacy studies and to inform animal numbers. Studies will be staged to allow data to be reviewed between animals as the efficacy work progresses.

Cadavers will be shared with other groups where possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs and sheep have been selected to appropriately test the system with regards blood flow, blood volume and a whole systems biological approach. The procedure is anticipated to cause minimal pain and adverse events are predicted to be seen quickly, while animals are still under anaesthesia hence optimisation will be performed under terminal anaesthesia. Once the protocol is defined and refined, recovery studies with general anaesthesia will be started.

Local anaesthetic may be used wherever possible, for example, to reduce stress prior to

taking blood samples.

Why can’t you use animals that are less sentient?

Adult large animal models are required for the safety testing of the system due to the necessary blood flow, blood volume and large enough access points via the cannulae in the veins.

Terminal anaesthesia studies are being used for the pilot study to refine protocols prior to starting the safety study and will be used for the efficacy studies involving inflammatory compounds. Terminal aneasthesia is not appropriate for the longer safety studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Prior to surgery, recovery animals will be trained as appropriate to the species, to co-operate and tolerate procedures, thus minimising stress during handling for observation / conscious procedures. Training may include clicker training and/or food rewards.

Post-operative care will include regular monitoring, including overnight care where necessary to ensure animal recovery is optimal and welfare of the animal is maintained. All animals will receive appropriate peri-operative pain management during and after the procedure. Surgical expertise at the test facility further enhances animal welfare, by providing close collaboration with dedicated large animal surgeons and veterinary anaesthetists, and ready access to advice in the case of unforeseen complications or intercurrent illness. For clarity on care in such circumstances, we will refer as appropriate to the NVS, in the first instance.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein. (Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs.)

Animals will continually be monitored for signs of pain and distress, especially post-challenge, by experienced veterinarians and animal care technicians with significant experience in these species.

Anaesthetists work to best practice guidelines for large animal anaesthesia and maintain CPD to keep up to date with new practices.

Standard Operating Procedures are employed for animal preparation, surgery and recovery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs are regularly published on internal News Forum and other relevant information

is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

201. Molecular Imaging 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, imaging, metabolism, 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 overall aim of our research is to develop non-invasive and clinically applicable molecular imaging methods that could be used in the clinic to detect the presence of cancer and cancer progression, for example in breast and ovarian cancer models, and to monitor the early responses of tumours to therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A growing understanding of tumour biology has led to the development of more targeted therapies, where a drug is designed to disrupt a specific cellular process, such as a signalling pathway, that is responsible for the sustained proliferation and survival of the cancer cell. However, the DNA mutations that drive activation of these pathways vary between different cancers and between cancers of ostensibly the same type. For example,

there are ten different types of breast cancer, which are distinguished by their underlying mutations and that have differing prognoses and treatment responses. In glioblastoma, a very aggressive brain tumour, DNA analysis identified four different types, with differing prognoses and in ovarian cancer differences in the numbers of copies of some genes have been shown to predict overall survival and the probability of drug-resistant relapse. The challenge is to match the drug to the tumour, targeting the specific processes driving cell growth and proliferation in that specific tumour type.

For the past 25 years my lab has been developing clinically applicable imaging methods, some of which have translated to the clinic, that could be used to detect the very early responses of tumours to treatment. These could be used in an image – treat – image – treat way to allow rapid selection of the most effective treatment for an individual patient. Since different drug combinations and the timing of their administration can affect efficacy, these imaging methods could be invaluable in selecting the most appropriate drug combination and administration schedule. Furthermore, most patients entered into early phase drug trials have advanced disease, which have already failed conventional therapies, and are arguably the most challenging group in which to ascertain activity signals from a new drug. Imaging methods that give a very rapid indication of treatment response could potentially be used to test drugs in “window of opportunity” studies. This would involve giving a new drug for a short period and imaging early response before standard-of-care treatment/management is initiated.

We have also started to explore the possibility that underlying mutations in tumour DNA can be inferred from pre-treatment image features, which can then be used to predict disease prognosis and drug sensitivity. Such information can also be obtained from tumour biopsies, however imaging has the advantage over biopsy in that it can more readily detect heterogeneity (both within and between tumours) and by imaging tumour biochemistry and structure may be a more effective way of selecting drugs than identifying tumour mutations.

The aim of our pre-clinical research over the next 5 years is to further develop our methods for detecting early evidence of tumour treatment response and to develop new imaging methods that can determine disease prognosis and which can be used to predict drug sensitivity.

What outputs do you think you will see at the end of this project?

My lab is focussed on clinical translation of the novel imaging techniques that we are developing. This is exemplified by the work that we are doing on the development of techniques for monitoring tumour metabolism using magnetic resonance imaging (MRI). Changes in tumour metabolism following treatment can give a very early indication of whether a patient is responding to the treatment. In the future this could allow a clinician to rapidly select the most effective treatment for an individual patient. Rapid onset of tumour cell death post treatment is also an indicator of a positive treatment response.

We are developing an agent that by binding to dying tumour cells allows their detection using positron emission tomography (PET), which is another imaging modality that is widely used in the clinic. The work that we are proposing to do under this new licence will continue to focus on the clinical translation of the novel imaging techniques that we are developing to detect treatment response. We will continue to publish our work in high profile journals and to present this work at major international meetings. In the last 5 years, work conducted under the current licence has led directly to 27 publications. In addition, I

have presented this work at 40 international meetings.

Who or what will benefit from these outputs, and how?

The focus of our work is the early detection of tumour treatment response with the aim of developing imaging methods that could be used in early phase clinical trials to get an indication of drug efficacy and subsequently in the clinic to guide treatment in individual patients. We are working primarily on three tumour types: Glioblastoma (GB) is the most common malignant primary brain tumour, which is increasing in incidence. The disease has a dismal prognosis. With the current standard of treatment, which involves maximal debulking surgery followed by chemoradiation, patients have a median survival of just 15 months. Treatment has not advanced significantly in the last 20 years and as a consequence GB is regarded as a cancer of unmet need. Ovarian cancer has an incidence/mortality ratio (approx. 7000 cases per annum and 4000 deaths) that is similar to lung and pancreatic cancer. The lack of robust and biologically validated imaging biomarkers for the disease leads to poor assessment of treatment response in routine clinical practice and in clinical trials. Breast cancer is a much more common disease, with nearly 60,000 cases per annum in the UK, and although much more treatable, with nearly 80% of patients surviving 10 years from diagnosis, there is still a significant mortality rate with nearly 12,000 deaths per annum. With all of these cancers we anticipate that the introduction of the novel imaging methods that we are developing will accelerate the introduction of new and more effective treatments for these cancers into the clinic. We will also do some work with xenografts of established pancreatic tumour cell lines where the goal is to develop imaging agents that can detect early evidence of disease.

How will you look to maximise the outputs of this work?

My laboratory is participating collaborative EU programmes have work packages and personnel dedicated to dissemination of the results of this work. In addition, the work coming out of my laboratory is regularly publicised in the popular press as well as being published in the scientific literature.

Species and numbers of animals expected to be used

- Mice: 3200
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We use mice because of the availability of models of breast and ovarian cancer derived directly from patients. These models are widely recognised as being useful models of the human disease. The models derived directly from patients display the same DNA mutations that are found in the tumours from which they were derived, but they lack an effective immune system. Importantly, from the perspective of this project, the tumours derived from patients show variable responses to standard-of- care and targeted therapeutics that reflect their underlying DNA mutational profiles. We use rats mainly for brain tumour models, where the larger size of the brain and resulting tumours are more compatible with the limited sensitivity and spatial resolution of the imaging techniques that

we use, which are clinically applicable techniques such as MRI and PET.

Typically, what will be done to an animal used in your project?

The experiments involve implanting tumour cells, which grow to form a solid tumour, and are good models of the human disease. Implanted tumours can take anywhere between two weeks (implanted murine lymphoma) to up to a year (implantation in the brain of a patient-derived model of glioma) to reach a size at which we would start imaging treatment response. Tumours implanted subcutaneously may be surgically removed to allow the development of metastases. Any animal that shows signs of distress or overt signs of sickness will be killed immediately by humane methods. However, this is very unlikely with the models that we propose to use. The animals will then be imaged using the new imaging agents and non-invasive imaging methods that we are developing, before and at various time points after administration of a new or existing medicine or therapy, in order to determine whether we can detect the early effects of treatment, for example evidence of tumour cell death. The imaging methods, which include magnetic resonance imaging, X-ray CT scanning, radionuclide imaging, ultrasound and optical imaging, cause minimal discomfort to the animal. Typically, the animals are anaesthetised for the imaging examination and a contrast agent, radiolabelled molecule or a cell preparation (e.g. specially modified immune cells) expressing a label that can be detected by non- invasive imaging may be administered, usually intravenously. The anaesthesia is used primarily to stop them moving during the imaging examination. Imaging examinations can take up to 5 hours, but typically are 2 hours or less and for some imaging exams, for example bioluminescence imaging, may be as short as 15 minutes. The animal is then allowed to recover from the anaesthetic and maybe imaged on further occasions at various times during subsequent drug treatment or therapy. At the end of this procedure the animal is killed using a humane method and the tumour tissue will usually be excised for biochemical and histological analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals experience minimal discomfort from tumours implanted under the skin. Animals are expected to experience moderate severity in relation to the surgical procedures, where these include implantation of tumour cells into the brain and surgical removal of established implanted tumours. Analgesic agents will be administered as required. For tumours implanted under the skin these will be monitored daily by visual inspection. For abdominal tumours or tumours implanted in the brain these will be monitored using non-invasive imaging techniques. Animals will be killed if they show signs of illness, where these include hunched posture, inactivity, neurological signs, for example difficulty in walking, or if weight loss exceeds 15% of body weight from the maximum recorded for the animal. The in vivo imaging methods we use are all well established and these methods and the reagents involved are not anticipated to have any adverse effects. Mild anaesthesia is used during imaging for restraint. For scans longer than 15 min body core temperature is monitored and maintained. Depth of anaesthesia is controlled by means of a respiratory pillow linked to a system reporting breathing rate. Animals will be rehydrated as required, for example, by injection of dextrose/saline.

Expected severity categories and the 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 considered to 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?

Any new imaging agent will usually be first tested on cells grown in culture. Only if it is demonstrated to work in this simple system will we progress to experiments on animals. Much of our work involves imaging tumour metabolism and although computer models of metabolism have been in existence since the 1970s these cannot reproduce the varied metabolism of the different tumour types that we work on, especially when this is modulated by the frequently chaotic and inconsistent tumour vasculature. Although the imaging techniques that we use, such as MRI and PET, are non-invasive most of the techniques that we are developing involve injection of an imaging agent and therefore it is not possible to transfer the novel imaging techniques that we are developing directly to the clinic. The animal work is essential to demonstrate the utility of the imaging agents. Without this information we could not get ethical approval for clinical studies, nor would we be able to raise the substantial funding required to conduct these patient studies.

Which non-animal alternatives did you consider for use in this project?

Any new imaging agent will usually be first tested in vitro.
Why were they not suitable?

In vitro systems cannot reproduce the influence of the circulatory system and the complex environment within a tumour on the delivery of the imaging agent to the tumour and its subsequent clearance.
Computer models do not capture adequately this complexity nor the complexity of the varied metabolism in the tumour types that we are studying. Direct transfer to the clinic is not possible since we need to demonstrate utility in animal models for ethics applications and to be able to apply for the substantial funding required for human 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 total number of mice and rats is based on initial experiments with groups of 4-5 animals (more may be used to establish statistical significance if warranted by these initial experiments) and the specific projects listed under Scientific Background.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Typically, we investigate a new imaging technique in a relatively small group of animals (4 – 5). As the imaging techniques are non-invasive and can be repeated up to 5 times in any one animal each animal can act as its own control, for example before and after treatment. This greatly improves the significance of any effect that we observe since it overcomes any variation within the cohort. For example, we find that the pre-treatment levels of cell death in some tumours are highly variable and can be greater than in some tumours post-treatment. By evaluating each animal individually, before and after treatment, as would be the case if we translated the technique to the clinic, we can get a more robust result from a smaller group of animals. After such an initial experiment we then consult the Institute's biostatistics unit for a statistical review of the data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The imaging techniques that we use are non-invasive, causing minimum discomfort to the animal, and therefore we are able to image tumours in individual animals over prolonged periods of time. This both reduces the number of animals that we need to use and also improves the quality of the data since each animal serves as its own control, minimising the effects of interindividual variability. The information content of any experiment is also maximised by taking tumour tissue at the end of the experiment for a thorough histological and biochemical analysis, which as well as revealing further insights into the biology of the tumour can also be used to validate the imaging findings. Experiments will be reported in accordance with the ARRIVE 2.0 guidelines (Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 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.

We use mice because of the availability of patient derived models of breast and ovarian cancer, which have both been developed in immune compromised mice. These models are widely recognised as being useful models of the disease. We use rats mainly for brain tumour models, where the larger size of the brain and resulting tumours is more compatible with the limited sensitivity and resolution of the imaging techniques that we use, which are clinically applicable techniques such as MRI and PET.

The imaging techniques and the majority of the surgical procedures (e.g. tumour implantation) are minimally invasive and thus are not expected to have any significant adverse effects.

We have introduced delivery of radiotherapy using a device that allows image-guided targeting of radiotherapy to the tumour and thus reduces normal tissue damage.

Previously we administered estradiol to promote the growth of hormone sensitive breast tumours via pellets implanted under the skin. We have now supplemented this approach with administration via the drinking water.

Why can't you use animals that are less sentient?

The rat and mouse patient-derived tumour models are widely regarded as useful models of cancer and there is an extensive literature on their use. Since we are interested in translating our techniques to the clinic for detecting treatment response, and these models have been shown to reproduce the drug responses observed in the clinic, we believe that these are the most appropriate small animal models to use. We do use terminally anaesthetised animals for follow-on mass spectrometric imaging studies on rapidly frozen tissue sections, which help us to interpret the in vivo imaging data as well as being important experiments in their own right for investigating tumour metabolism in vivo.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

My laboratory conducts multi-modality imaging research (magnetic resonance imaging (MRI), x ray computed tomography (CT), positron emission tomography (PET), ultrasound, photoacoustic, bioluminescence) and therefore we are well placed to detect the early growth of tumours that cannot be palpated, such as intra-cranial glioma patient-derived xenografts and intra-abdominal ovarian patient- derived xenografts. We can also use imaging techniques such as MRI, ultrasound and CT to detect the development of metastases. Moreover, we frequently genetically modify implanted tumour cells so that they express an enzyme that produces light when provided with its specific substrate, which enables rapid screening for tumour growth using bioluminescence imaging. These techniques allow us to select animals for imaging research projects before they manifest clinical signs of disease. The imaging techniques are minimally invasive, requiring only light anaesthesia to prevent animal movement during the exam. Animal suffering is minimised by appropriate use of anaesthetics and analgesics.

The use of the SARRP to deliver targeted radiation minimises normal tissue damage, with welfare benefits for the animals. For example, we have not observed skin ulceration when delivering radiation treatment using the SARRP.

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, P., Aboagye, E.O., Balkwill, F., Balmain, A., Bruder, G., Chaplin, D.J., Double, J.A., Everitt, J., Farningham, D.A.H., Glennie, M.J., Kelland, L.R., Robinson, V., Stratford, I.J., Tozer, G.M., Watson, S., Wedge, S.R., Eccles, S.A. & An ad hoc committee of the National Cancer Research, I. (2010) British journal of cancer 102, 1555-1577.

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.).

Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18(7): e3000410

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are ably supported by a highly professional staff in our Biological Resources Unit, who send out, via email, regular updates from the NC3Rs as well as informing us about NC3Rs workshops and online videos. In addition, our work under this project licence will undergo a thorough mid-term review by our Animal Welfare and Ethical Review Board, which further ensures that we are up-to-date and implementing any advances in the 3Rs.

202. Novel Immuno-oncology 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, Therapy, Tumour, Animal Models, Immuno-oncology

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 our project is to develop novel immune-therapies to treat cancer. A new treatment has to be safe and effective. Lead compounds will be tested in animal models of cancer, such as, ectopic tumours, acute myeloid leukaemia, and patient derived xenograft models for their ability to reach their target tissue from their site of administration (Bioavailability), modulate the immune response to a tumour and provide a clinically measurable beneficial effect when compared to a vehicle (Efficacy) or when compared to a reference drug (Potency) while having limited side effects (Safety).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 test the ability of the lead candidate to prevent or cure cancer or how it compares to existing reference drugs, we may perform efficacy or potency studies in animal models. These types of studies will typically compare the effect of a prophylactic (prior to the clinical signs being established or to the administration of the disease-inducing agent, or cells) and/or therapeutic administration (after clinical signs are established) of a lead candidate to the effect of a vehicle administration and/or reference drug. When a new model is identified or when the mechanism of action of the lead candidate justifies an assessment in an efficacy model not currently validated by us, efficacy model validation studies will be performed.

New approaches to cancer research are constantly developing, further amendments to this licence may be required in the future in order to insert new models, to modify existing protocols or adding novel therapeutics and therapeutic approaches according to new state-of-the art findings in the literature.

What outputs do you think you will see at the end of this project?

New data and knowledge will be generated in the area of immuno-oncology. This will help drive development of novel anti-cancer therapies.

Data outputs

The data generated under this project will provide information on lead compounds' safety, efficacy, bioavailability and potency (efficacy against a reference drug):

This work is expected to provide new information on the beneficial effect of lead compounds when compared to a vehicle.

New information on beneficial effect of lead compounds when compared to a reference drug (A reference drug is defined as a clinically relevant drug which has been granted authorisation by the centre of the Medicines and Healthcare Products Regulatory Agency (MHRA)) or control group. The data generated will typically be sufficient for the study sponsor to decide the lead compound’s future: (i) pre-clinical toxicology, the next step in the drug discovery process leading to a novel therapy (ii) additional efficacy studies or (iii)

rejected for lack of safety or efficacy.

Who or what will benefit from these outputs, and how?

Throughout the life of this project we will generate new information that will provide a new understanding of mechanisms of immuno-oncology therapies as well as give an indication of efficacy of novel immuno-oncology therapies. These will be fed back to our client to inform the next stage in the drug development process. We will also promote any refinements or best practice we identify during this project. We will make post-mortem tissue available to groups we collaborate with. In the medium term the pharmaceutical industry will be interested in potential novel therapeutic targets we identify.

The long-term potential benefits of this study are that data generated may have far-reaching implications for the treatment of cancer, both in humans, benefitting patients and clinicians.

How will you look to maximise the outputs of this work?

We will maximise outputs by using the knowledge gained by running the models routinely to advise future clients on study design, appropriate power and how the model can be refined to reduce suffering whilst maintaining scientifically meaningful data.

Some data may be used to generate marketing literature. These data are made available to the scientific community (i) during scientific conferences we attend, (ii) upon request through our website or
(iii) through targeted email campaigns. Moreover, some of our clients do use the generated data for scientific publications.

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 used as the standard model for tumour efficacy models as they are easily genetically manipulated and a large number of diverse tumour types are available and validated; This allows comparison of novel therapies against the existing large volume of historic data testing cancer therapies. For immune targeted therapies the mouse has a mature immune system or can be repopulated with human immune cells.

Models described in this license have been developed in adult mice and therefore only adult mice will be used under the protocol listed. The methods discussed in this license have been published in peer- reviewed journals by academic or industrial groups. The models have been used to test the efficacy of drugs currently on the market.

Typically, what will be done to an animal used in your project?

Animal models for oncology studies will be obtained by either administering murine or

patient-derived tumour cell lines via intravenous, intraperitoneal or subcutaneous route of administration in order to promote the growth of different types of tumours.

Lead compounds used to test efficacy and substances used for disease induction will be administered by one, or a combination of different routes of delivery, typically, oral gavage, intravenous, subcutaneous or intraperitoneal injections.

Animals will be monitored for generalised clinical signs, tumour volume and location and bodyweight loss.

Average duration for most of the protocols described in this license ranges between 6 and 12 weeks. However, studies can last from 1 week to 14-16 weeks depending on the model.

Specific guidelines for cancer research will be followed as summarized in the paper from Workman et al., Br. J. Cancer (2010): 102 p1555.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals might show bodyweight loss due to tumour development and after administration of the lead compound.

Animals will be monitored regularly for tumour formation, growth and occurrence of adverse effects like generalised clinical signs including changes to body condition, abnormal posture (e.g. hunched), abnormal coat condition (e.g. piloerection), abnormal breathing, abnormal movements, decrease or increased activity in response to stimuli.

Expected severity categories and the 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 are 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 system's response involves multiple systems, multiple organs and multiple cell types. The complexity of the immune response and its interaction with the multiple cell types within the tumour microenvironment cannot currently be reproduced in vitro.

Which non-animal alternatives did you consider for use in this project?

In vitro experiments on tumour cell lines or patient-derived material (PDX) co-cultured with immune subsets from healthy human donors and ex-vivo experiments on cell cultures prepared from tissue sampled in animals humanely killed will be performed to assess various parameters and study some aspects of the immune response

Why were they not suitable?

The complexity of the immune response cannot be completely reproduced in vitro. In addition, the symptoms of cancer cannot all be modelled in vitro. We will, however, endeavour to perform in vitro studies on tumour cells where applicable. We run these assays as co-cultures using human tumour cells and immune cell subsets from healthy donors to model anti-tumour responses. Where a more complex systems-based approach is required, in vivo models remain the only way to assess efficacy and mechanism of action of a drug. One of the subsidiary aims of the project is to explore whether we can generate more complex in vitro models to recapitulate the tumour microenvironment and whether these can be used in vitro as a valid alternative to some in vivo immuno-oncology models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on previous studies run at our site we have determined the number of animals that we need in each study to determine whether a particular treatment has an effect or not and anticipate running one such study per year.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Research staff are trained in Experimental Design and Statistical Analysis of Biomedical Experiments. We have made use of local support including our institutional statistician and other resources such as the ARRIVE guidelines and 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?

Where suitable, previous experimental data from our establishment and others will be used to allow for comparison. In addition, if possible, we encourage the use of a shared control group among different studies using the same model.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models described in this licence have been developed in mice and therefore only mice will be used under the protocols listed. Animal suffering will be limited by using the mildest disease inducing agent or dose, and studies will be kept as short as possible. Animals are monitored frequently for signs of discomfort, and appropriate action taken promptly. We will monitor animals closely throughout the studies, and they will be treated or humanely killed if they develop signs of excessive suffering

Why can’t you use animals that are less sentient?

Mice are the lowest sentient species that can be used for the purpose of this project. While some individual aspects of tumour formation can be modelled in vitro, the complexity of the interactions between multiple body systems that is critical to understanding immune-oncology and define treatment efficacy cannot be replicated in vitro.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In general, animal suffering will be limited by ensuring that the models used cause the least harm to the animals. The mildest disease inducing agent or dose will be used, and studies will be kept as short as possible. Animals are monitored frequently for signs of discomfort, and appropriate action taken promptly. We will monitor animals closely throughout the studies, and they will be treated or humanely killed if they develop signs of excessive suffering.

In more detail:

Exploratory tolerability studies are short-lasting experiments which require small numbers of animals of the same species that will be then used for tumour experiments. These studies establish if a lead compound generates adverse events without having to use a larger number of animals. Adverse events are limited in duration by monitoring the animals frequently and culling animals showing more than moderate and transient changes to the normal appearance or behaviour.

Pharmacokinetic studies establish if a lead compound has sufficient bioavailability prior to the lead compound being administered to a larger number of animals. Adverse events are limited in duration by monitoring the animals frequently and culling animals showing more than moderate and transient changes to the normal appearance or behaviour.

Efficacy models listed in this license are validated models. The methods have been published in peer-reviewed journals by academic or industrial groups. The models have been used to test the efficacy of drugs currently on the market.

In all efficacy models, the intensity and duration of adverse events are limited by not

allowing more than moderate changes to the normal appearance and behaviour of the animals. Animals exceeding this limit are culled immediately. Adverse events are identified early by (i) starting to monitor the animals immediately after disease-induction and prior to the expected onset of clinical signs, (ii) monitoring the animals frequently and (iii) increasing the monitoring regimen at times of peak disease. Animals are monitored for disease-specific signs (e.g. tumour formation) but also for non-specific clinical signs such as changes in appearance (posture, piloerection) and behaviour (spontaneous or provoked).

Animals are housed in groups and kept in an appropriate environment with plentiful bedding and nesting material and suitable objects that allow them to express normal behaviour. All staff are trained in good animal handling procedures. Animals are always handled gently and humanely, especially animals which may be in pain. Animals may be acclimatised prior to being handled before the experiment starting so that they are less stressed once the study begins. Cupping and tube handling are used routinely as our method of handling.

Animals are provided with a bowl of mashed food on the cage floor if moving may be uncomfortable. When substances need to be administered, we will give the smallest volume possible and administer it in the way that causes the least distress.

Moderate signs are not tolerated for more than 24 hours and severe signs will not be tolerated. At the end of an experiment, all animals will be humanely killed to enable further in vitro testing of samples.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will refer to the guidance on experimental design from the NCR3s. In addition, we will perform power calculations based on our control data from previous studies and studies performed at other Charles River sites and allowing for anticipated effect size of novel therapies. In addition, we and our clients will refer to published literature in this field to help determine the effect of size of therapies directed against similar therapeutic targets or cell types.

Moreover, specific guidelines for cancer research as summarized in the paper from Workman and colleagues, published in 2010 will be followed. In more detail, we will integrate the guidelines published by Workman and colleagues concerning study design, statistics and pilot studies; choice of tumour models (e.g., genetically engineered, orthotopic and metastatic); therapy (including drugs and radiation, when appropriate); imaging (covering techniques, anaesthesia and restraint); humane endpoints (including tumour burden and site); and publication of best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Information gathered through continued professional development and courses, literature, liaison with our NVS, scientific conferences and NC3Rs and institutional resources will be used to improve the way we run our projects and maintain best practice.

203. Evaluation of new devices to be used in human surgery

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

Surgical devices, Endoscopy, Laparoscopy, Robotics

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?

This project aims to evaluate and further develop novel surgical devices and to identify those which have the potential for use in human surgery. Then to progress these devices, from the bench testing phase, through the necessary pre-clinical regulatory assessments to be registered by, for example, the Medicines and Healthcare products Regulatory Agency (MHRA), the European Medicines Agency (EMA) and/or the US Food and Drug Administration (FDA) for human 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?

All devices which are intended for human clinical use need to undergo rigorous safety and efficacy assessment before progressing from pre-clinical to clinical use. Some of these assessments are to establish which of several potentials is the most appropriate to progress to patient use and some are to definitively establish safe use of the final chosen instrument. All devices under this licence are intended for use in human surgery and are designed to increase positive surgical outcomes, enable new surgical approaches and/or minimise the impact of the surgery itself.

What outputs do you think you will see at the end of this project?

The devices successfully evaluated will be made available to clinicians and surgeons worldwide.

These evaluations should allow rationalisation of potential devices, to defined products, to progress through relevant regulatory bodies, to human clinical use and be used to inform the next generation of a device or new ideas for novel energy delivery.

'Energy delivery' includes the use of microwave-, radio- and ultrasound-frequencies, all of which can be used in patient diagnosis and/or treatment by focusing them in different ways. For example, ultrasound is currently commonly used as a safe, standard method of monitoring a baby's health in the womb however, it is also possible to use ultrasound to treat patients - by using a different form of ultra sound, high intensity focused ultrasound (HIFU), it is possible treat conditions such as uterine fibroids or gall stones.

Several minimally invasive devices (including those for endoscopic, laparoscopic and robotic use) could be registered for human clinical use over the course of this licence.

Most of the work carried out under this licence will probably be for device development therefore publication may be limited by the companies' intellectual property concerns and, as such, it may not be possible to publish it, although there now seems to be an increasing interest in publishing data from such studies.

Who or what will benefit from these outputs, and how?

Benefits should be felt immediately upon the release of devices for clinical use, leading to feedback and the next generation of devices thereby providing long-term benefits to patients who currently need repeat or additional treatments which can extend their dependence and treatment regime.

These evaluations may provide safe additional, or replacement, devices (or combinations) for patients with varying conditions. Many of which could be applicable to several conditions from wound care to complex surgical interventions and thus improve the lives of a wide spectrum of patients.

These benefits may improve surgical outcome thereby improving quality of life for patients together with a reduction in surgical procedure duration, and the related anaesthetic requirements, as well as recovery time and therefore length of hospital stay, which would also decrease NHS costs and free up much-needed bed space. In some cases, these new devices may allow surgical intervention where currently it is not possible, thus increasing the options open to patients with several different medical conditions. For example, previous advances in endoscopic tool and technique development have changed patient treatment from open bowel surgery (requiring significant theatre time and several days

hospital stay) to endoscopic surgery (which can be done as a day case, requiring no overnight stay).

How will you look to maximise the outputs of this work?

Where possible, publication in peer-reviewed journals, dissemination at national and international meetings, workshops and seminars. Also, getting new, effective devices/compounds into clinical use as quickly and safely as possible will maximise patient benefits and healthcare savings.

Species and numbers of animals expected to be used

- Pigs: 470

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 device testing, we need an animal of a similar size to humans as the devices to be tested will be the same size as those intended for human clinical use. Adult pigs are therefore the animals of choice for these evaluations due to their size and anatomical similarities.

Typically, what will be done to an animal used in your project?

Devices will be evaluated for safety and efficacy.

Initially these studies will be non-recovery and only devices showing good results will be carried through to recovery studies. Some of these results may be obtained with the aid of endoscopic or minimally invasive surgery and/or the use of non-invasive imaging such as X-ray, MRI or ultrasound.

For the recovery studies, this will be done by the anaesthetised animal undergoing surgery in a similar way to that of a human patient. The effects would also be monitored in a similar way, for example using a combination of the following: observation of general health and behaviour, regular blood tests, non- invasive imaging (e.g. x-ray, ultrasound or MRI) or via further endoscopic assessment to visualise the operated area and potentially to also take biopsies. It may be possible to carry out ultrasound and even, in some cases, x-ray without the use of anaesthesia however, the other imaging/assessment options would require general anaesthesia both to enable precise imaging and positioning but also to reduce stress for the animal. Also, as with human patients, the benefit/necessity of the various monitoring modalities will be weighed against their impact on the patient.

For example, for an endoscopic access to the bowel for simulated polyp removal, under recovery anaesthesia an endoscope would be introduced via the anus and navigated to the appropriate area of the intestine (it may be necessary to flush the intestine at this point to remove any faeces present). The area would be marked, an endoscopic injection used to raise a 'polyp' and the mucosal tissue excised (approximate area: 3-6cm in diameter), this may be repeated several times (to replicate the human clinical situation). The

treatment sites would then be checked and the scope removed and the animal allowed to recover from the anaesthesia. Blood samples may be taken pre- and post-operatively as well as at several later time points to assess any change in general blood chemistry. Possible re-scoping time points for an eight week study would be days 0, 3, 7, 14, 28 and at termination this would allow a visual assessment of the treatment site- any necessary blood samples would also be taken at these times. These blood tests and scoping 're-looks' would also serve as a method of monitoring overall animal health and any significant deviations from normal that could cause unnecessary animal suffering would constitute a humane endpoint. As these animals would have no external wounds or cannula there should be little or no need for individual housing. Where possible, blood sampling, scoping and minimally invasive imaging will be carried out at the same (or at least overlapping) time points to minimise the number of instances of anaesthesia the animal has to undergo thereby stressing the animal as little as possible. This should mean that, for a 4 week study, there would be no more than 6 instances of anaesthesia including the initial surgery and at termination.

At the end of the experiment the animal will be humanely killed and the operated site examined and removed for further examination by a pathologist.

What are the expected impacts and/or adverse effects for the animals during your project?

For endoscopic procedures:

From previous experience, we do not expect to see any adverse events. Following complete recovery from anaesthesia the animal should appear normal and show no signs of pain, weight loss, or abnormal behaviour.

For laparoscopic procedures:

Potential adverse effects

From previous experience, and because all devices we need to test will have undergone rigorous bench testing and, where possible testing on dead tissue, we do not expect to see any adverse events. However, post-surgical infection is always a possibility following surgery. This will be specifically monitored and, if it does occur, relevant intervention or treatment under veterinary advice, will be applied. If intervention or treatment is inappropriate or ineffective the animal will be killed using a humane technique.

Avoidance of adverse effects

Good sterile technique and good preparation of the subject before surgery will ensure the absolute minimal chance of infection. Good use of pain relief medication will minimize the possibility of anything more than minor discomfort associated with the surgical procedures. Good monitoring for signs of pain will allow timely intervention under veterinary advice.

For open procedures:

Potential adverse effects

From previous experience, and because all devices we need to test will have undergone rigorous bench testing and, where possible testing on dead tissue, we do not expect to

see any adverse events. However, post-surgical infection is always a possibility following surgery as is wound dehiscence/ suture failure. These will be specifically monitored for and, if it does occur, relevant intervention or treatment under veterinary advice, will be applied. If intervention or treatment is inappropriate or ineffective the animal will be killed using a humane technique.

Avoidance of adverse effects

Good sterile technique and good preparation of the subject before surgery will ensure the absolute minimal chance of infection. Good use of pain relief medication will minimize the possibility of anything more than minor discomfort associated with the surgical procedures. Good, multi-layer surgical closure will reduce the risk of suture line failure. Good monitoring for signs of pain will allow timely intervention under veterinary advice.

Humane endpoints

If any animal shows unacceptable changes to behaviour or physiology, full consultation with the vet and other local animal welfare staff will be undertaken to determine the best clinical care for the animal. If improvement is unsatisfactory 24-48 hours after any intervention or treatment, the animal will be killed using a humane 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)?

Pigs:
Non-recovery - 32% Moderate severity - 68%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 assessment of safety, effectiveness of surgical devices, requires full biological systems to show that they do not cause any unacceptable reactions in living tissue but that they have the appropriate effect in as 'human-like' an environment as possible. It is not yet possible to accurately and reliably simulate this complete system.

Which non-animal alternatives did you consider for use in this project?

We have considered virtual reality for testing of the devices and we do, where possible, use these systems and simulations for ergonomic testing and early instrument development. We use tissue from dead animals for all initial tests where possible, but ultimately we need to know that the material or material/instrument combination is safe

and effective in a fully functional biological system.

Why were they not suitable?

Virtual reality systems are not currently safety or efficacy predictive and as such they are not yet accepted by regulatory bodies. And it is not possible to assess healing, or the ability to stop bleeding, in dead tissue.

It is not, currently, possible to carry out full device testing without using an in-vivo model as there is a need for a complete biological system, especially if looking for histological and/or haematological responses (including testing of coagulation efficacy) and/or longer-term healing. Using pigs for device testing has several well established models which we have used for many years; due to their anatomy and general size/weight, they are currently the most appropriate for testing devices, prior to their use in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For all safety and efficacy studies we will reduce numbers as far as possible by utilizing data as predicate where devices/materials are similar and by using as many sites as possible per animal without impacting upon the data obtained or negatively affecting the animal's welfare.

The number of animals is based upon previous device testing studies we have carried out over a number of years where the number of animals used was based on the advice from bio-statisticians. For any significant changes to protocols, further statistical advice may be sought.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible tissues are retrieved from animals killed following the completion of other studies to reduce the number of animals used for tissue retrieval and instruments will have been progressed through assessments using dead tissue, prior to progressing to full evaluation in a live model. We will continue and expand this philosophy to reduce the numbers of animals needed in the pre-regulatory studies and follow the Norecopa PREPARE checklist. Norecopa is Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement) in connection with 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?

As we retrieve more knowledge from these studies and build an "in house" data bank we will be able to refer to these results thus reducing the numbers 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.

Surgical devices need to be assessed in an appropriately sized animal whose anatomy mirrors that of humans as closely as possible and the pig is typically used for this, especially with respect to the abdomen and gastro-intestinal tract. Safety and efficacy evaluation of the instruments and material/instrument combinations needs to take place in as close a situation to human use as is possible. Some models have been developed by us over several decades of device testing and others can be taken from the literature but there may be some incidences where a model will have to be developed to adequately and specifically answer the study questions. Any models developed to test instruments or materials will take direct note of the potential for discomfort to the animal. As these evaluations are to facilitate translation from pre-clinical to clinical human cases we feel there is little point subjecting an animal to a procedure which would not be well tolerated by a human subject and, as with human subjects, anaesthesia and pain relief will be used to minimise pain and discomfort.

Why can’t you use animals that are less sentient?

For the device testing we need an appropriately sized animal as the devices to be tested will be those intended for human clinical use. The pig is therefore the animal of choice for these evaluations. Also, the majority of the initial testing will be carried out on animals that never recover once anaesthetised at the start of the experiment.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The use of best surgical practice and adherence to the principles set out in the LASA (Laboratory Animal Science Association) guiding principles document combined with good pre- and intra- operative care and monitoring will minimise unnecessary suffering. The use of minimally and non-invasive assessment (e.g. MRI or X-ray) as well as scoping, whilst increasing the number of anaesthetics an individual animal has over the course of a study, can significantly increase the amount of information gained per animal (by allowing internal assessment at multiple time points) and therefore reduce to overall number of animals used. Also, with a degree of animal 'training' and familiarisation, and the correct pre-medication (often delivered in food rather than by injection), the stress/suffering to the animal can be minimised - this applies to medication delivery, acclimatisation to single housing, blood sampling from a cannula and any other events that require interaction with the animal. Time spent 'training' each animal also allows the animal husbandry staff to become more familiar with the personality of each animal and therefore more aware of any changes in behaviour (which is most often the first sign of any systemic change). Also, following open abdominal surgery, the use of a mash/ more liquid diet to aid digestion

immediately post-surgery is often recommended.

By combining as many procedures as possible, it should be possible to reduce the number of anaesthetic events each animal undergoes.

Again input/ support from the local NIO, NACWO, NVS and other local animal care staff will greatly help with this.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Norecopa PREPARE and NC3R and ARRIVE checklists coupled with reviews of the current literature and any revisions to the regulatory guidelines along with reference to the LASA guidleines on undertaking aseptic surgery (2017 edition). I have also been referred to standard, established, well regarded reference books, for up to date anaesthesia advice/techniques.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Review of the current literature and any revisions to the regulatory guidelines along with input from the local Named Information Officer (NIO), Named Animal Care Welfare Officer (NACWO), Named Veterinary Surgeon (NVS) and other local animal care staff. As well as checking the Norecopa, NC3Rs and LASA (and similar animal research and welfare) websites

Home Office

204. Improving sustainable parasite control in ruminants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- (d) Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Parasitology, Immunology, Epidemiology, Diagnostics, Control

Animal types	Life stages
Cattle	juvenile, adult, pregnant
Sheep	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 programme of work is to improve our understanding of the immune responses of ruminants to natural exposure with parasitic diseases in the UK with a view to providing farmers, veterinarians and other industry stakeholders with tools to improve disease control, welfare and productivity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parasitic diseases of ruminants, predominantly sheep and cattle, disproportionately impact pasture- based production systems. This is important, as pasture-based production is considered a sustainable and environmentally friendly long-term solution for UK food producers. Emergence of resistance amongst parasite populations to, and the environmental impacts of commonly used parasiticides further complicates this issue, meaning additional and alternative control measures need to be developed and implemented to allow long-term and sustainable parasite control in UK ruminant species.

What outputs do you think you will see at the end of this project?

New information

The primary objective of this project is to improve our understanding of host-parasite interactions between UK ruminants and their common parasites. This will ultimately be of benefit by allowing us to advance sustainable parasite control measures for UK ruminants. This is important as parasite control in the UK and more generally is becoming increasingly problematic due to widespread emergence of drug resistant parasite populations, changing weather patterns which favour the development and transmission of several parasitic species.

Publications

We anticipate this new information and insight into parasitic diseases of ruminants, which will result in a number of scientific publications in relevant peer reviewed journals. For example, we expect the work to yield at least 2 publications, whilst the anticipated follow-on work into bovine parasitic bronchitis is expected to yield at least one additional scientific publication. Additional research intended to be carried out under this licence (e.g. genetics of hair breed sheep on parasite resilience) are also expected to yield further scientific publications.

Products

Work carried out under this project licence will also yield products that will be of direct benefit to sustainable parasite control efforts in UK livestock in a number of ways through development of new and improved options for diagnostic testing and identification of potential vaccine candidates.
Furthermore, epidemiological data collected under this project licence will aid in the evaluation of additional supportive tools for sustainable parasite control such as predictive risk models and diagnostic surveillance networks with the ultimate aim being to incorporate these into sustainable parasite control programmes.

Who or what will benefit from these outputs, and how?

This work will benefit the scientific and academic community by improving our basic understanding of important parasitic diseases of livestock and the veterinary and farming communities through translation of this improved understanding into improved methods for disease control, ultimately improving UK food security and sustainability.

How will you look to maximise the outputs of this work?

In addition to scientific publications, knowledge transfer of relevant findings will be disseminated to the relevant stakeholders through relevant channels such as farming press and animal health groups. For example, a project on lungworm partnered with a prominent small-medium enterprise who specialise in knowledge transfer on animal health matters in the livestock sector to farmers, vets and animal health care professionals. Relevant findings will also be disseminated through the relevant steering groups for sustainable parasite control in sheep and cattle.

Species and numbers of animals expected to be used

- Cattle: 2500
- Sheep: 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.

Sheep and cattle are natural hosts for the parasitic diseases we are proposing to investigate under this project licence and therefore most appropriate species to use for the current project, particularly since it is our principle aim to investigate responses and dynamics of natural exposure and challenge. Parasitic infections typically affect juvenile animals, but can also impact adults. Since commercial livestock are predominantly kept for production purposes it is also likely adult animals may be pregnant when sampled. The data collected by using an approach of sampling commercial animals under normal production conditions will make our findings of direct relevance to UK farming and veterinary communities.

Typically, what will be done to an animal used in your project?

Animals will be gathered using the standard livestock handling system by normal staff at the POLE for a period not exceeding 1-2 hours. Sampling will require manual restraint for a period not expected to exceed more than 30 minutes after which they will be released back into their normal management group. Following sampling, any animals requiring sedation will be penned separately to other stock where possible and provided with fresh water. They will be checked periodically until such time as they have recovered sufficiently to re-join their usual management group.

What are the expected impacts and/or adverse effects for the animals during your project?

1. Subcuticular haematomas may result from jugular venepuncture.

2. Minor rectal tears may occur as a result of rectal sampling for faeces.

3. Fractious behaviour following placement of a nasal tube to collect bronchoalveolar lavage samples.

4. Bronchoalveolar lavage may worsen the condition of dyspnoeic animals by further reducing already limited lung capacity.

Expected severity categories and the 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 all cases, likely incidence of this occurrence is low.

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?

Use of animals is essential for the success of the project and required for all three programme objectives.

Where possible, we will seek replacement by using samples collected either for clinical diagnostic purposes and/or at post-mortem. This will be possible when initially establishing a number of our proposed in vitro assays and will also allow us to obtain some initial immunological data as described for objective 1. However, since our work aims to determine the dynamics of immune response associated with natural challenge within and between herds and over time, the sampling of live animals will be a necessity for objectives 2 and 3. In most instances this will be limited to collection of blood and faeces as described in protocol 1. For objective 3, serology and faecal testing represent the predominant methods for conducting parasitological diagnosis and surveillance. It may be possible to replace protocol 1 in some instances by collecting faecal samples from the floor (as described in the proposed work flow in protocol 1). It may also be possible to reduce the requirement for serum sampling by adapting diagnostic tests to use milk samples, which will be evaluated as part of objective 1.
However, for some animals (e.g. young stock, beef cattle etc) and assays this may not be possible.

In the case of pneumonic parasitic diseases, such as bovine parasitic bronchitis (infection with *Dictyocaulus viviparus*), additional sampling is proposed through bronchoalveolar lavage (BAL) as described in protocol 2. This represents an opportunity to evaluate local inflammatory responses to lungworm infections through a minimally invasive sampling method. Previous research have only characterised local immune responses through post-mortem collection of lung tissue from experimentally infected animals. Whilst it will be possible for us to meet objective 1 by replacement of some sampling of live animals with samples collected post-mortem, this will simply not be an option for sampling of animals in commercial herds when investigating objective 2.

Which non-animal alternatives did you consider for use in this project?

Bovine alveolar and intestinal epithelial models have been developed for use in studying bacterial and protozoal pathogens *in vitro*

Why were they not suitable?

Small single cell morphology infection models are not an appropriate tool for study of macroparasites as proposed here.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For each step we will aim to produce statistically robust results that allow us to detect biologically relevant processes using the minimum number of animals. Numbers of animals required are dependent upon the aims of specific objectives (e.g. immunological versus epidemiological studies) and the varying methods by which minimum sample sizes are calculated for these different studies (described under experimental design).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will minimise variation within groups by selecting control animals from the same POLEs as infected animals, aiming to match breed, age and sex where possible. As discussed under replacement, we will also aim to reduce the number of animals that need to be sampled under protocol 1 by developing diagnostic assays that use milk and through use of fresh faecal samples collected from the floor rather than per rectum where possible. All in vitro experiments proposed for this project will be performed either in duplicate or triplicate to reduce the need for re-sampling and discard of inconclusive data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?
Principally, reduction will be based on the use of sample size and power calculations to determine minimum sample size for a proposed sensitivity and power of 95% and 80%, respectively with effect size determined by use of relevant data from previous immunological and epidemiological studies. The proposed PPL holder has personal experience with such calculations for immunological investigations, with is additional statistical support for this purpose 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.

We intend to use commercially bred sheep and cattle from UK farms (POLEs) naturally exposed to the parasitic diseases of interest to us. These animals will be handled and sampled using standard farm equipment and veterinary techniques with severity of proposed procedures kept to a minimum. To reduce suffering and distress further, where possible sampling will be carried out at the same time as routine farm procedures to reduce the number of times animals need to be gathered and handled.

Whilst in poorly managed stock parasitic diseases can be a significant source of mortality, morbidity and reduced animal welfare, in many cases animals harbouring infections will not show obvious clinical signs. We anticipate that in the majority of cases we will be working with clinically healthy animals in the majority of cases, but in situations where clinical disease is observed this information will be fed back to the farmer along with any useful diagnostic or surveillance data resulting from our studies. This will improve the information available to farmers and their veterinarians concerning the on- farm disease status of their herd/ flock.

Why can’t you use animals that are less sentient?

Sheep and cattle are natural hosts for the parasitic diseases we are proposing to investigate under this project licence and therefore most appropriate species to use for the current project, particularly since it is our principal aim to investigate responses and dynamics of natural exposure and challenge.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where possible, sampling of cattle and sheep on farms will coincide with the farm’s usual management practices, for example, routine milking, whole herd/ flock treatments etc. Collection of blood will be no more invasive than routine diagnostic testing and will be conducted as described in protocol 1 to minimise risk as much as possible, including limiting the frequency and volumes for collection.
Collection of bronchoalveolar lavage (BAL) samples (protocol 2) will use the minimum quantity of fluid required (50mL) to reduce the risk of adverse effects. This procedure is routinely used to diagnose and evaluate respiratory conditions of young calves in veterinary practice. In protocol 2, we include the need for clinical examination of any dyspnoeic animals by an experienced livestock veterinarian before BAL sampling is authorised.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs website updates

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attendance of regular workshops and webinars organised by the establishment, Home

Office and/or external organisations such as NC3Rs.

205. Bone Marrow Transplantation: Biology and Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants.

Key words

No answer provided

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Treatment methods that use one's own immune system to treat cancer have been extremely beneficial in recent years. However, progress has been limited with respect to blood cancers, which include diseases such as leukaemia, which is a debilitating disease that requires complicated treatment procedures. Blood cancer patients are treated with BMT that both cures the cancer and provides the individuals with a brand-new immune system. In most cases, patients who undergo BMT develop disease conditions such as graft versus host disease (GVHD) while undergoing treatment. GVHD is an immune condition that arises following a transplantation procedure between the same species. The adverse reaction occurs when the donors' immune cells, known as T cells, attack the tissues of recipient (host). The hosts' tissues are attacked as the donors' T cells do not recognise the different proteins, known as antigens, within the tissues. GVHD is a debilitating disease that causes significant death in patients undergoing BMT. Additionally, some treatments have only limited anti-cancer response (Graft versus tumour effect; GVT). Using mouse models for understanding the biological problems associated with GVHD and GVT and testing novel therapies for GVHD and GVT is an extremely important field of research for both children and adults who suffer from blood cancers. The objectives of the current proposal would involve understanding the basic biology of GVHD and GVT in a mouse model of BMT. Mice will be given a treatment schedule that closely mimics that

which patients with blood cancer receive. The transplanted mice will then be treated with new medicines that minimise GVHD while maximising GVT. These studies will help in addressing the dire clinical needs that are currently required for better treatment of blood cancers in children and 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.

What are the potential benefits that will derive from this project?

The proposed study will advance the field of bone marrow transplantation in many different ways including the development of cutting-edge cancer treatment strategies, which reduce the detrimental effects that the cure (cell transplant) can have on the patient. This study will also understand the basic biology of GVHD, which will result in developing new methods of transplantation that can prevent GVHD but maintain anti-cancer effects for patients.

Species and numbers of animals expected to be used

What types and approximate numbers of animals will you use over the course of this project?

We will use mouse as a species for the purpose of this project. We will minimise the number of animals used in any one experiment by using careful statistical analysis and appropriate control treatment groups. Depending on the data generated, trials that show promise may need follow-up studies with greater numbers of animals (typically no more than 20 per group). We predict that we will use 17500 mice in order to obtain meaningful results from our treatment regimens.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?

Since the objective of our research is to utilise the immune system to cure cancer, the first step is to introduce a new immune system into the mice. This new immune system will be derived from donor mice. Animals will receive bone marrow cells from donor mice that have a completely different immune system. This will result in host mice developing a strong immune response that will be treated with medicines that can decrease side effects from giving these transplants.

The adverse effects that the animals may suffer will be limited due to the experience of the investigator with these animal models. However, a few adverse effects can be predicted. A major complication of GVHD will be weight loss, animals that do lose more than 25% of their body weight will be humanely killed. However, immediately after the transplant, animals will be monitored daily until their weights are stable and fluids will be provided. Another complication of transplantation could be infections. Animals will be treated with

antibiotics two days prior to the transplant and will be maintained on antibiotic water for the rest of the period of the experiment (usually 21 days). Since the animals will also be transplanted with a relevant tumour, the size of the tumour will be monitored daily and animals will be humanely killed before it could have an impact on welfare. Animals will also be humanely killed if on examination, if the animals were moving slowly due to the tumour. The animals will also be provided with appropriate diet meals to combat any dehydration prior to and post transplantation.

Animals are expected to show no more than moderate severity and will be humanely killed if deemed to be in danger of exceeding this.

At the end of the procedure, after the animals are humanely killed, their organs will be removed to study their response to cancer.

Replacement

State why you need to use animals and why you cannot use non-animal alternatives.

Pilot data has been obtained that suggests that immune therapeutic strategies work in preventing GVHD but also in treating GVT. However, the interaction of the various parts of the immune system cannot be understood from in-vitro assays and requires the use of whole animal models. Relevant data using predictive mouse studies will therefore establish the maximum benefit and efficacy of these agents as novel therapeutics for human cancer and inform the appropriate design of clinical trials in man.

In order to determine non-animal alternatives to study the effect of immune cells in GVHD and anti- tumour biology, we have performed a thorough literature review of the field. Standardised and widely accepted web engines such as Pubmed and Web of Science was utilised for this literature search.
Mostly available alternatives to in-vivo animal studies are in-vitro experiments. Unfortunately, in-vitro experiments do not capture all the interaction that occurs between the various immune cells and tissue derived cells which are vital for understanding how immune responses are modulated in GVHD. Therefore available in-vitro techniques are not ideal for fully understanding the function of immune cells in a disease such as GVHD. However, we will still use in-vitro and ex-vivo assays where appropriate.

For eg., invitro immune cell based assays will be used to test the optimal drug and radiation doses to be used in-vivo. These dose response in-vitro assays will enable us to use appropriate concentration of drugs that will produce maximum anti-GVHD response or engraftment response with minimal discomfort for the animals. These in-vitro assays will also enable reducing the number of animals to be used since the dose response will not be done on animals. It will also reduce unwarranted adverse effects which may be associated with higher doses of radiation and/or chemotherapeutics.

Reduction

Explain how you will assure the use of minimum numbers of animals.

We will utilise the minimum number of animals based on appropriate statistical calculations that will provide maximum information on whether the treatment has an effect in providing

anti-cancer responses. Hypotheses and experimental designs will be designed to minimise the numbers of mice used and the duration of experimental studies.

In the first instance we will use power calculations to determine animal cohort size for each study performed within this licence. The power calculation for in-vivo studies is outlined. For in-vivo analysis, the experimental unit is the mouse. We are planning a study of a continuous response variable from independent control and experimental mice with 1 control(s) per experimental mice. In a previous study the response within each group was normally distributed with standard deviation 7. If the true difference between the cohorts is 11, 7 experimental cohorts and 7 control cohorts are required to reject the null hypothesis with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. For experiments with two groups, statistical analysis will include a two-tailed student t test and for experiments with more than two groups, a one-way ANOVA analysis will be performed.

In addition we will also use the tools available to us through the NC3Rs website. We will use the online experimental design assistant available within the NC3Rs to determine sample size. All the data generated from mouse studies will be reported in accordance with the ARRIVE guidelines.

Refinement

Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.

In this project licence, we will use mice as the model system. The reason for using mice stems from the observation that the immune system of both human and mouse are closely related. Mouse models of human diseases are a good representation of human disease and is widely accepted by the scientific community. Many medicines that have shown efficacy in mouse disease models have been successfully used to treat patients in a clinical setting. The research to be conducted in this project will aim to understand the biology of BMT, which is the most complicated immune therapy available for treatment of blood cancers. It is not possible to study the changes that occur in the immune system during BMT by using standard cell culture assays. It is also not possible to set up clinical trials in order to optimise drug doses for BMT. Hence, a mouse model of BMT is the most refined system for this research project due to the similarities in the immune system while providing a context for immune cell interactions in the living body after BMT. Moreover, the availability of reagents that help study mouse immunology is extensive as compared to other larger animal models or non-human primates. These benefits will help in the progress of BMT research which will result in the development of better clinical trials for patients with blood cancers.

Specific animal models which are the most refined for understanding BMT biology and therapy are as follows

Mouse model of radiation: The dose of radiation or chemotherapeutics that provide optimal stem cell repopulation in the body after BMT is not completely understood. Identifying optimal doses for radiation or chemotherapy will prevent unwarranted side effects such as radiation induced toxicity and other side effects which are notoriously associated with high chemotherapy doses. Dose response studies cannot be performed using in-vitro cellular assays or through clinical trials. Therefore, the mouse model shown here is the most

refined for this purpose. In order to prevent unnecessary adverse events, we will take a number of precautions whereby high radiation doses are split into multiple doses so the animals do not suffer unnecessarily with long-term radiation exposure.

Opportunistic Infections: A number of opportunistic infections can be manifested in patients who undergo BMT. It is important to understand exactly how infections can affect various cells of the tissue and the immune cells in BMT. As previously mentioned, in-vitro assays or studying immune cells from infected patients will not fully capture the extent of damage caused to both tissue and immune cells all within the context of the infectious pathogen. Therefore whole animal models are the most refined in order to study the biology of infection related tissue and organ damage in BMT. Further refinement techniques will include using appropriate infectious doses such that animals will experience minimal distress in this protocol. Furthermore, it is not possible to test new drugs for infectious complications directly in patients but this can be efficiently performed in animal models. Taken together, the mouse is an appropriate model for studying this process.

Graft versus host disease: A major complication associated with BMT therapy is acute GVHD which occurs within 100 days post transplantation followed by chronic GVHD. These processes are characterised by systemic inflammation thereby it is not possible to study the trigger that causes GVHD by using cellular in-vitro assays or through blood samples from patients. Therefore, the mouse model of GVHD is vital and the most refined to understand this biology. Furthermore, this model will enable the development of new therapies that will be beneficial for patients who suffer from this debilitating disease. Therefore mouse is the most refined model that can be used. In addition, we will ensure that animals receive the minimal amount of cell dosing in order to develop disease and will be treated with appropriate medicines on disease development. We will not allow animals to succumb to disease for long periods of time and animals will be closely monitored in order to minimise pain and discomfort.

Treatment of drugs will be done such that appropriate minimal doses are used.
Tumours: Animals with tumours will be closely monitored and the impact of tumour cells on immune cells within the tumour microenvironment will be studied. Unfortunately, we cannot fully understand this biology using in-vitro assays or from tissues in tumour patients. This is because once the patient develops tumour it is not possible to study the triggers that intiated the disease. Hence mouse models are the most refined to study tumour biology. Refinement measures will include dosing mice with minimal number of tumour cells that will result in optimal tumour burden without causing unnecessary pain and distress. Additionally, mice will be monitored closely to determine any distress caused by tumour burden and this will be immediately rectified through interventions. Further refinement measures will include treating mice with chemotherapeutics at minimal doses that provide anti-tumour response but does not cause other debilitating effects or pain or distress to the animals.

In addition to these measures, within the whole project licence, further refinements that will be applied in all studies are expanded below.

Animal suffering will be minimised by monitoring mice under experimental protocols to detect distress. Experiments will be terminated once the objectives and end points have been collected. Animals will be subjected to anaesthesia prior to invasive procedures and suitable post-anaesthetic care will be provided. Humane endpoints will be applied to minimise suffering in each model used in the study. A summary of endpoints are as

follows:

Mice will be humanely killed if they lose more than 25% body weight loss from that of initial weight. We will closely follow the clinical scoring sheet to monitor the health and well-being of the animals during the course of the experiments outlined in the respective protocols 2-6.

Supportive care will be provided to mice in danger of losing significant body weight as dietary supplements. Animals that look unwell will be monitored closely for signs of dehydration, weight loss and piloerection. In case weight loss cannot be reversed within a reasonable timeframe (e.g.,7 days) and reach more than 25% body weight loss from initial weight, the animals will be humanely killed.

Mice will be treated with antibiotics immediately prior to and post BMT.

Mice bearing tumours >1.5cm^3 mean volume in any direction (2cm^3 volume for therapy groups, but mice will be monitored daily from 1.5cm^3) will be humanely killed in accordance with published guidelines.

Home Office

206. Sensory processing in teeth

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

prevention, management, neuropathic orofacial pain, phantom tooth pain, irreversible pulpitis

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to develop murine models for inflammatory tooth pain (irreversible pulpitis; aka classical ‘toothache’) and for neuropathic orofacial pain (phantom nerve tooth pain). Following the development and validation of these models, we intend to investigate mechanisms underlying peripheral and central processing of tooth pain, before exploring treatment methods that will lead to the improvement of tooth pain control in humans and 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 most common cause of pain within the face area in the UK is acute inflammatory dental pain, colloquially called “toothache” (8-12% of population per year). This condition may result in irreversible inflammation of the tooth’s nerve and blood-vessel tissue (= dental pulp) leading to a condition called irreversible pulpitis (permanent “toothache”). This

pain can be currently treated by removal of the dental pulp by one of two treatments: root canal therapy or extraction of the affected tooth. Given the nature of these procedures, both treatments may sometimes lead to the development of ongoing chronic neuropathic (nerve) phantom tooth pain. This has been reported to occur in 1.6% of cases post-treatment, but the biological mechanisms behind this transformation from an acute ("toothache") to chronic (nerve) pain are not well understood and therefore are difficult to prevent. Given the NHS in England alone performs approximately 3.1 million extractions and 600,000 root canal treatments per year in adults, this represents a substantial problem and therefore there is a pressing need to improve our understanding and knowledge of these mechanisms to reduce the risk of chronic pain development. Furthermore, current treatment options involving medications for both irreversible pulpitis (permanent "toothache") and phantom tooth pain (chronic nerve tooth pain) are not very efficient, so it is hoped that furthering our understanding will lead to novel approaches that will improve the management and control of this pain.

There are only a few animal models available for these tooth pain conditions. However, none of them addresses the complexity of pain processing associated with these conditions. There have been no robust animal models for either irreversible pulpitis or chronic nerve tooth pain developed. This has led to a lack of understanding of the mechanisms that generate and maintain the pain symptoms of both acute dental pain and (its conversion to) chronic tooth pain. Unsurprisingly, this means, there is an extremely limited number of pain control/prevention strategies available. Those that are available tend to focus on managing pain with systemic medications that may be associated with many side-effects. The nature of the tooth anatomy and those two conditions would allow applying medications locally, directly into the source of pain, which would presumably be associated with less of side-effects, but this strategy has not been extensively studied.

Thus, the purpose of the proposed study is to establish novel pre-clinical models of irreversible pulpitis and chronic nerve tooth pain to define the complexity of the molecular mechanisms underlying these distinct, but common, pain conditions in humans. We plan to use a chronic pain model alongside the acute inflammatory tooth pain model as the rationale basis for therapeutic intervention in acute dental and chronic nerve tooth pain, developing novel therapeutic strategies for both conditions. We will also address the possibility of understanding of potential benefits associated with a local application of medications.

What outputs do you think you will see at the end of this project?

The proposed research will provide us with important and novel animal models allowing us to identify the complexity of mechanisms that underlie the development and management of acute inflammatory ("toothache") and chronic neuropathic (nerve) tooth pain. Most importantly, it will allow us to develop novel therapeutic strategies for the improvement of tooth pain control in humans. It will also improve the understanding of mechanisms of pain sensation that will help to improve the welfare of animals in the context of general pain management in animals but also when animals may experience similar pain as part of other models and/or procedures.

A number of management strategies are currently employed to manage toothache, which has a limited or absent evidence base. Some of these approaches involve the use of an antibiotic/corticosteroid paste and may give a moderate improvement of pain. We hope that the outcomes of the proposed research will benefit future medical management by reducing the unnecessary use of antibiotics (therefore reducing antibiotic resistance) whilst

having direct benefits in pain management and the prevention of developing chronic nerve tooth pain. For patients (and animal models) that develop chronic nerve tooth pain, we will also focus on further exploration of topical application of medications. This will have important clinical benefits by reducing side-effects from systemic management and could lead to new products being available commercially. Thus, our findings will be of interest to all health care professionals who deal with patients suffering from facial pain but ultimately, it is the patients who will be the true beneficiaries.

Our studies will address both genders as female rats are rarely used in pain research. Publishing our data involving both male and female rats will contribute to the evidence base in using both genders, and ultimately lead to a reduction in female rats being euthanised.

Who or what will benefit from these outputs, and how?

Poor understanding of dental pain mechanisms and lack of effective treatment is compounded by the lack of robust animal models to investigate the processes involved in irreversible inflammatory (permanent “toothache”) pain and its transition to chronic neuropathic (nerve) tooth pain. Our current work developing a lab based in vitro model of pulp (the centre of a tooth) tissue will form one part of studies to understand this process, but it also requires the simultaneous development of a comparative in vivo models to allow the development of potential therapies to cross the first translational gap into tests in patients. This may allow us to improve our management of irreversible pulpitis and chronic nerve tooth pain in a way which can have direct positive effects by reducing patient pain, reducing the associated impact upon the quality of life and improving the maintenance of pulp vitality, therefore, reducing the need for further treatment.

Short term benefits. Understanding irreversible pulpitis, chronic nerve tooth pain and their management in animal models will offer indications for new treatment approaches in humans.

It is intended that based on the outcome of the irreversible pulpitis model, a randomised controlled trial can be proposed to compare the management of this condition using different strategies.

Identifying novel treatment strategies for chronic nerve tooth pain is equally important, with current approaches offering moderate control of symptoms only. A better understanding of how this chronic condition develops, may immediately affect how endodontics (part of dentistry focusing on root canal fillings) and extractions are carried out. Furthermore, it will enable randomised controlled trials to be considered, something which our establishment is uniquely positioned to deliver.

Publishing our proposed models will mean work can be undertaken at other establishments, opening the door to a wide range of investigations on the management of these two conditions. This would be very important as currently available models are not a true reflection of those conditions.

Long term benefits. The long-term primary benefits of both models are to improve patient outcomes. For irreversible pulpitis, it is hoped that based on collected evidence we will be able to offer pain control without the unnecessary use of currently available medications that seem to have limited therapeutical benefits and prevent the transition to chronic nerve tooth pain. The management of chronic nerve tooth pain is equally varied and probably

less effective than that of irreversible pulpitis. Identifying new applications for current medications or novel therapies, will improve the outcomes for many patients. Understanding mechanisms for how chronic nerve tooth pain is initiated will also help inform best practice for extractions and root canal fillings.

Secondary benefits are the reduced cost of treatment for both conditions. For irreversible pulpitis, opening opportunities for alternative treatments which aim to keep the tooth alive, would avoid the
time-consuming and expensive requirement for root canal fillings, or the financial and functional burden resulting from an extraction. Avoiding the unnecessary use of e.g., antibiotics would reduce global microbial resistance, having obvious benefits for all patients. Reducing the risk of developing chronic nerve tooth pain following irreversible pulpitis, root canal treatment or extraction would avoid a potentially long-term condition with currently poor treatment outcomes after a protracted period of management. Directly linked to this last point are improved outcomes for chronic nerve tooth pain, which would have economic benefits by reducing treatment duration and reducing the oral health-related impact on the quality of life.

How will you look to maximise the outputs of this work?

This project represents internal and external collaboration between our institution and other institutions. Each institution brings unique expertise to maximise the potential basic science and translational gain from the projects. This combines extensive experience of pain models in rodents with the clinical expertise of dental pain, commercial links and access to cohorts of patients with irreversible pulpitis and chronic nerve tooth pain.

The outcomes of the study proposed within this application will represent a novel model for irreversible pulpitis and chronic nerve tooth pain that we aim to publish in peer-reviewed journals and present at national and international scientific meetings. Innovative management approaches will be explored with potential for clinical and commercial translation.

Species and numbers of animals expected to be used

- Rats: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

9-11-week-old Sprague Dawley rats will be specifically used in this project as the root formation of the tooth is almost complete by this age and therefore suitable for proposed interventions. The nerve structure of the tooth is also nearing completion at 10-weeks, suggesting maximum maturity of the pulp. Beyond this point, work on the dental pulp becomes increasingly difficult due to the deposition of reactionary dentine. Nerve innervation also decreases beyond 12-weeks. The selection of the age of animals for this project is further supported by our own work based on analysis of trigeminal neurons cultured in the laboratory environment that has been undertaken in 10-week-old rats. It has been established that in vitro (laboratory) techniques become less reliable in older rats. Moreover, age matching between our in vivo and in vitro models would align and

strengthen our research outcomes.

Mice cannot be used for our proposed models. Through work on both mouse and rat cadavers, the clinical procedure of causing a communication into the dental pulp followed by setting up and inflammatory/infective process (replicating irreversible pulpitis) cannot be predictably undertaken in the mouse molar. The tooth and pulp space is too small, and there is insufficient space to place a dressing into the pulp chamber.

Typically, what will be done to an animal used in your project?

Objective 1 and 3: Objectives 1 and 3 are to understand how pain is regulated in an animal model of irreversible pulpitis (‘toothache’), followed by determining the mechanisms involved in its current management approaches and novel approaches to produce relief from this pain.

For objective 1, after acclimatisation period, rats will be subjected to a pulp injury e.g., in their upper first molar. This procedure will be undertaken under general anaesthesia and using aseptic techniques discussed and agreed with the named veterinary surgeon. Post-operative analgesia may be given (non-steroidal anti-inflammatory drugs - NSAIDs). The pulp injury will lead to bacterial contamination and the development of irreversible pulpitis over a 2-7 day period. The animal will then be euthanised and tissue from the nervous system that is associated with pain processing will be used in biochemical assays to determine mechanisms underlying irreversible pulpitis.

For objective 3, animals will be subjected to similar procedures as described above but will then have topical treatments applied to the pulp e.g., antibiotic to understand the importance of individual components of current treatment regimes. Animals will be assessed for their pain thresholds before and after the treatment. The animal will be euthanised within 14 days of induction of the pain state.
NSAIDs will be administered post-operatively. Although there is concern that pain-relieving medication may affect our study of pain in the models, there is evidence that this may not be the case. Human patients with both irreversible pulpitis and chronic nerve tooth pain would usually take NSAIDs and the results from the study will therefore have greater translation if systemic pain relief is used in the models.

Objective 2 and 4: Objective 2 is to understand how pain is regulated in chronic nerve tooth pain characterized by long-lasting pain in a tooth or teeth, or in a site where teeth have been extracted or following root canal treatment. Objective 4 is to understand the mechanisms underlying current management approaches and novel approaches.

For objective 2, after acclimatisation period, rats will be subjected to a pulp injury e.g., in their upper first molar. A paste of known neuropathic (pain-inducing) agents (e.g., glutamate, substance P and CGRP; these are mediators which are typically found during pain and inflammation in humans and rats) will be applied to the pulp. This will lead to the development of chronic nerve tooth pain. NSAIDs will be administered for post-operatively. After a period of maximum 12 weeks, the rat will be euthanised and tissue samples will be processed to identify mechanisms underlying this pain condition. The acute phase of pain is unlikely to last beyond 7-days as the tooth will become non-vital (will die).

For objective 4, the chronic nerve tooth pain will be managed, acutely or chronically, with the administration of treatment e.g., local application of gabapentinoids (used to treat nerve pain in humans). Animals will be assessed for their pain thresholds before and after

the treatment. The rat will then be euthanised after allowing the treatments to take effect to identify mechanisms underlying the effect. This will vary depending on the management strategy employed.

What are the expected impacts and/or adverse effects for the animals during your project?

Normal recovery from anaesthesia is expected within 1-2 hours post-surgery. In some cases, <1% of animals may require up to 4 hours to return to normal behaviour. Uncommonly, animals may fail to recover from anaesthesia within 4 hours. These animals, <1%, will be observed up to 24 hours and subjected to a programme of enhanced care until the animal reaches full recovery. Any animal not showing normal recovery post 24 hours will be humanely killed.

After induction of the tooth pain conditions, temporary weight loss may be observed; typically: < 10% throughout the experiment. If weight loss exceeds 10% then additional measures will be taken to rectify the situation. Animals will not take part in any experimental procedures until recovery has taken place. If >20% with no clear signs of improvement following fluid replacement and/or medical treatment advised by NVS, the animal will be humanely killed.

After induction of the tooth pain conditions, animals will develop a localized inflammatory response that may include swelling and tenderness in areas including and surrounding the site of surgery. Animals giving any cause for will be removed from the study until they recover. Supportive treatment will be provided. If any adverse effects persist for a period of 24 h the animal can be humanely killed after NVS/NACWO's recommendation.

Expected severity categories and the 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 75-85% of animals are likely to experience 'moderate' severity. This is because they will undergo surgery to induce a pain state (either irreversible pulpitis or chronic nerve tooth pain), after which they will receive systemic pain relief. For the irreversible pulpitis model, the pain will be limited to 2-7 days whilst the condition develops followed by up to 2 weeks treatment for the condition. For the chronic tooth nerve pain model, the acute pain is likely to last up to 7-days, after which the tooth will normally become non-vital (will die). It is planned to administer pain relief (NSAIDs) post-operatively. Any residual pain after this stage will likely be a result of the development of persistent pain. These animals may also be subjected to repeated behavioural testing and dosing of substances. The remaining 15-25% of animals are likely to experience mild severity because they will not undergo the surgical procedure. These animals may still be subjected to repeat behavioural testing and dosing of substances depending on the experimental design and type of controls required.

The severity is anticipated to be 'moderate' from the experiment. However, as these are new models of pain, this will be reassessed during the development of the models. It is planned to work closely with the NACWO/NVS to re-evaluate severity after 6-8 animals subjected to each protocol. If indicated, an amendment will be submitted to increase the rating to 'severe'.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We know that the sensation of pain is supported by complex central nervous system (CNS) networks and ascending/descending pathways that are conserved across mammalian species including humans. Fortunately, previous research in rodents has shown that these animals can shed considerable light on human diseases, including pain, and indeed even lead to new therapeutic approaches. The proposed models aim to more closely replicate human models than existing animal models, allowing therefore for research with greater translational potential.

We will use rats because these species have informed us quite accurately about many aspects of pain processing in human and they are well-characterized models, that are used widely in nervous system research. It has been demonstrated that the rat molar pulp responds to inflammation in a similar way to humans, and it also has repair potential that is seen in humans. Rats express pain-related behaviours e.g., allodynia, hyperalgesia and sensitisation thus making them a species of choice for pain research.

Rodent models have mostly replaced feline, canine or primate models. It is possible to use in vitro models based on the use of culture neurones (e.g., trigeminal neurons), however such models do not allow to examine the full complexity associated with the development and maintenance of pain and therefore any outcomes gained from the cultured neurons would not have the full translational potential. Thus, the culture neurons are very useful however, they cannot entirely replace the use of animal models as they are rather simplistic and we are studying complex diseases that require to look at the behaviour of the whole animal.

Which non-animal alternatives did you consider for use in this project?

Where possible, we use cultured sensory neurones, harvested from rat pups. This model provides an invaluable in vitro system for studying the molecular processes that underlie somatosensory signalling and pain. We are using cultured sensory neurones for identification of mechanisms and then we aim to map relevant in vivo rodent models to validate the in vitro results in line with our programme of work. In this aspect, cultured sensory neurones are very useful however, they cannot entirely replace the use of animal models as they are rather simplistic, and we are studying complex diseases that require to look at the behaviour of the whole animal.

Immature cells (human induced pluripotent stem cells (hiPSCs)) also represent useful research platform for in vitro neuronal condition modelling and exploration alongside potential preliminary drug screening. The use of hiPSC-derived models also helps to replace and reduce the involvement of animals in pain research. Amongst the obvious advantages of hiPSCs are their authentic human origin and they potentially could be

differentiated into any cellular type of interest including neurons of the central and peripheral nervous system by using suitable protocols. But again, this non-animal alternative is rather simplistic, and we are studying complex diseases that require to look at the behaviour of the whole animal.

We are also considering the use of an in vitro 3D model of dental pulp tissue using induced pluripotent stem cells which are differentiated into odontoblast and neurones (the two cells which make up the outer layer of the pulp tissue). We are considering this model to reduce the need for animal testing, but ultimately this model is not entirely suitable for this purpose.

In addition, we intend to explore neuronal changes by using electrophysiological analysis. However, this model needs to be validated, and seeing similar neuronal changes in the animal model neurons will enable further in-vitro work potentially reducing the use of animals in the future.

Why were they not suitable?

Pain is supported by complex CNS networks and ascending/descending pathways that are conserved across mammalian species including humans. We are studying complex diseases that require to look at the behaviour of the whole animal.
A 3D in vitro model could not be used to investigate these changes, especially since the complex interplay between ascending and descending pathways could not be replicated. Due to the early nature of this project in which stem cell science is still developing and the fact that it only involves two of the cells seen within the pulp tissue and none of the second order neurons which reside within CNS suggest that the 3D in vitro model could not be used to investigate the changes in its full complexity. It is ultimately hoped that this model although simplistic in form will be able to help identify promising therapeutic targets and reduce the need for toxicology tests in animals. However, this model requires an in vivo model for comparison to validate it which these in vivo models will allow. We have also been informed by relevant regulatory bodies that any drugs tested using an in vitro model will still require an in vivo model in animals even if it is just repurposing of a licenced medication proving the need for valid in vivo animal models of irreversible pulpitis and chronic nerve tooth pain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In designing our experiments, we will ensure the minimum number of rats are used in order to collect data to answer the research question/s. Where relevant, once preliminary results (from pilot studies) will be obtained, our group will make appropriate arrangements to randomly assign animals to experimental groups and to blind studies, and we will plan and conduct studies to enable them to be published according to the ARRIVE guidelines.

Our models aim to explore peripheral and central pain processing which means we are

aiming to gain maximum information from each experimental intervention. Therefore, the tooth/supporting bone and parts of the nervous system involved in pain processing like trigeminal ganglion and subnucleus caudalis will be removed from the rat for biochemical and histological examinations.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We currently do not hold preliminary data that could foster our experimental design in respect to reduction of the number of animals being used in this project. Based on extensive experience and the validated nature of the tests, n=6-8 animals are sufficient for determining significance for the behaviour and histology experiments.

For the irreversible pulpitis model, we will ensure the model is validated before moving on to explore potential treatment interventions. We will gain as much data as possible in order to do this, including histology centrally and peripherally (which will enable the confirmation of the presence of irreversible pulpitis and inflammatory processes associated with this), culture of nerve cells and electrophysiology in vitro (to identify changes of nerve cells), and gene sequencing (to identify the expression of genes suggesting the presence of inflammation). For the chronic nerve tooth pain model, the same steps of the investigation will be undertaken, with our primary focus on the development and validation of the model. This will confirm the reliability and validity of each model before moving on to experiments to investigate interventions aiming pain control. This will ensure that we will gather valid data from all animals during the experiment, limiting 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?

As both proposed models are novel, pilot studies will be undertaken during development and validation of these models. We specifically aim to start with 6-8 male rats and with irreversible pulpitis models before moving to other steps of the proposed project. Collected pilot data will inform power calculations (it is also intended to reassess severity of the models at this stage). An amendment may be considered here to increase severity to ‘severe’ in two instances. Firstly, working closely with the NACWO and NVS, if the animals appear to be suffering above the level of ‘moderate’ severity, and amendment will be applied for. Secondly, if the animals develop the histological signs of irreversible pulpitis, but changes are not seen centrally, we would reduce or remove the post-operative analgesia administered. In this instance, an amendment would be applied for to increase the severity rating to ‘severe’.

In exploring different treatment strategies, the results will be regarded as satisfactory when a clear conclusion emerges: either a statistically significant difference or a sample size (guided by the resource equation) that should have revealed a difference should one exist.

To maximise the use of rats and where relevant, after the completion of behavioural in vivo examinations and following the experiment end, structures involved in processing of irreversible pulpitis and chronic nerve tooth pain (e.g., the tooth/supporting bone, certain parts of the peripheral and central nervous system) will be extracted and tissue used to examine the biological changes associated with e.g., the development of pain or the effect of treatment aiming pain control. To that extent, we are reducing the number of animal models required by simultaneously investigating changes in the tooth and the peripheral/central changes associated with pain. Previous published work has often

focused on one or the other.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 proposed two models; one to investigate irreversible pulpitis and another to investigate chronic nerve tooth pain.

The first model (irreversible pulpitis) represents a refinement of previously published models, to more closely replicate irreversible pulpitis in humans and to address challenges reported in the literature relevant to the models. This will enable more accurate exploration of the impact of pain (peripherally and centrally) and the effects of any treatments (the same treatments may be administered to the rat as humans, something which is not possible in previous models). The pulp will be opened by drilling, and a ‘leaky’ filling will be placed in the tooth. This will enable rapid contamination of the pulp (replicating the mechanism seen in humans). It is anticipated that irreversible pulpitis will develop within 24-48 hours. Once the model is validated, interventions are planned at this stage to manage the pain, which will involve a second procedure to place medication/s onto the pulp. Pain relief in the treatment group should occur quickly and it is planned to investigate outcomes of interventions within 2-weeks to better understand the relevant process, although human models report relief of symptoms within 24-48 hours. Pain relief will be administered for the animals.

The second model (chronic nerve tooth pain) is novel and has not previously been explored. An agent will be placed on the pulp (nerve) of the tooth which will induce nerve pain. It is likely that acute pain will reduce within 7-days as the tooth becomes non-vital (will die). It is planned to administer pain relief as post-operative care. The model takes time to become chronic (usually 3-months in humans).
Following this, novel treatments aiming pain control will be explored.

We have refined the methods on cadavers to minimise the time taken for the procedure and ensure it can be undertaken consistently. Through our application of medicaments, we are expecting the pain from irreversible pulpitis and chronic nerve tooth pain to be alleviated.

The severity is anticipated to be ‘moderate’ from the experiment, as we plan to administer post- operative pain relief. However, as these are new models of pain, this will be reassessed during the development of the models. It is planned to work closely with the NACWO/NVS to re-evaluate severity after 6-8 animals subjected to each protocol. If indicated, an amendment will be submitted to increase the severity to ‘severity’.

Why can’t you use animals that are less sentient?

Younger animals cannot be used because their teeth are immature and not a good model for irreversible pulpitis or chronic nerve pain. At an early stage of development, the teeth have an increased blood supply and increased capacity to heal and resist infection. Furthermore, the pulp is immature, and its neural supply is not yet developed meaning the response to noxious stimulus will be different. Other less sentient species cannot be used as their teeth or dental pain sensory systems do not compare easily with that of humans. The mouse molar is too small to predictably cause a tooth injury and place a medicament within the tooth. We have undertaken the procedure on cadaver mice, and found it is impossible to cause a predictable pulp injury which will develop into irreversible pulpitis in a way which replicates the human disease process. It is also impossible to manage the disease in the same way it is managed in humans (by removing inflamed pulp and placing a dressing within the pulp space, followed by placing a temporary filling). This limits our ability to undertake the work and minimises the translational potential to human benefit.

Lower vertebrates (e.g., frogs) or invertebrate species (e.g., insects or crustacean) do not have teeth, and do not represent the complexity of the mammalian nervous system and the concept of ‘pain’ is poorly defined.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures to improve animal welfare and minimise suffering will include monitoring animals, providing the animals with a high-quality modern caging environment with stringent welfare monitoring and minimising stress associated with behavioural protocols by performing tests in quiet environment, after allowing animals to initially adapt to the tests and handling.

Proposed models of pain will be produced using surgical procedures that will be done aseptically with refined surgical techniques to minimise the risk of infection as guided by NVS. These models of pain may produce a moderate degree of increased pain perception and altered pain responses, characterised as increased sensitivity to mechanical or thermal stimuli, but no other significant alteration in animal behaviour is anticipated. All animals may experience some post-operative pain and discomfort because of the surgery. Soft bedding, wet mash, chew blocks, additional fluids and supplementary heat will be provided to help to mitigate the pain if necessary.

We also intend to use pharmacological methods to reduce the pain this will include Non-Steroidal Anti- Inflammatory Drugs (NSAIDs; e.g., Aspirin, Ibuprofen). Despite a concern, these may interfere with the induction and development of pain responses, there is evidence that this may not be the case.
Furthermore, humans will have usually taken such medication to ease their pain, thus increasing the translation potential of the research. Stronger analgesics may have an unknown effect on the central pain processing and have performed worse than a placebo for dental pain. It is therefore planned not to use centrally acting analgesics.

To monitor animal condition post-surgery, we intend to use our establishment’s post-operative monitoring sheets which records a number of parameters postoperatively. Staff at our institutions are familiar with this scoring system and will be able to give us guidance and advice. We will follow NACWO/NVS’s advice closely.

Body weight will be our primary concern and therefore it will be monitored daily, together with food and water monitoring. If weight loss exceeds 10% additional food and mash food

will be provided to rectify the situation. The animal will not take part in any experimental procedures until recovery has taken place. Doses of drugs to be tested, routes of administration and an administration schedule, will be chosen so as not to have adverse effects. The methods of assessing pain thresholds that we will use are widely used in the pain research field, and I have extensive experience working with them. Thus, the obtained experimental results will be relevant to our previous research work and to other laboratories using these animals in studies of pain worldwide.

To ensure welfare, animals will be monitored on a daily basis by PIL holder and/or authorised staff for signs of extreme distress/discomfort and such animals will be humanely killed after consultation with NACWO/NVS. Animals will be humanely killed after a period of no longer than 12 weeks.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will plan and conduct studies to enable them to be published according to the animal research: reporting of in vivo experiments (ARRIVE) guidelines. The reporting guidelines from the planning research and experimental procedures on animals: recommendations for excellence (PREPARE) will also be used; these guidelines compliment the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are fully aware of the ethical and legal considerations relevant to the use of animals for scientific purposes and we are actively implementing NC3Rs principles in our work. Importantly, our institution is internationally recognised where adherence to the NC3Rs principles is a primary focus of all animal care staff and researchers, and this attitude is impressed on every new member of the University via interactions with those staff.

In the view of the expertise at our institution,we aim to improve non-pharmacological methods of pain control and objective methods of assessment of animal wellbeing during the experimental procedures.

The National Centre for the Replacement, Refinement and Reduction of Animals in Research website contains relevant updated information, and also provides regular webinars to update researchers on current best practice.

Home Office

207. Understanding Ovine Pulmonary Adenocarcinoma for Veterinary and Human 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
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Ovine pulmonary adenocarcinoma, Disease transmission, Disease control, Comparative oncology, Lung cancer

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 first aim of this project is to understand the infectious process of the Jaagsiekte sheep retrovirus (JSRV). This knowledge is essential for developing ovine pulmonary adenocarcinoma (OPA) control methods which can be used in eradication programmes or assurance schemes. The second aim is to develop a model of OPA which can be used in translational studies for human lung cancer research. This model will be essential for novel drug and therapeutic/diagnostic device research and development 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?

OPA is an infectious, fatal lung cancer of sheep that is caused by the JSRV. Mortality rates

in OPA affected flocks can be as high as 20% with significant implications to farming efficiency, economic viability and animal welfare. The disease is endemic in the UK and in most countries where sheep are farmed. The lack of a reliable diagnostic test for identifying JSRV infection during early disease means that OPA often goes unreported and makes it difficult to estimate the full economic cost of OPA. Concerns over OPA have risen in recent years and there is a need for further OPA research to underpin the development of control strategies.

Lung cancer represents a major worldwide health concern; although advances in patient management have improved outcomes for some patients, overall 5-year survival rates are only around 15%. In vitro studies and mouse models are commonly used to study lung cancer and their use has increased the molecular understanding of the disease. Unfortunately, mouse models are poor predictors of clinical outcome and seldom mimic advanced stages of the human disease. Animal models that more accurately reflect human disease are required for progress to be made in improving treatment outcomes and prognosis. Similarities in pulmonary anatomy and physiology potentially make sheep better models for studying human lung function and disease. Similarities in lung size allow sheep models to be used in ways not available in mouse models; techniques including drug administration, advanced imaging, ultrasound, endoscopy and surgery can be used in sheep as they would be in humans. OPA shares several common features with human lung adenocarcinomas, including histological classification and activation of common cellular signalling pathways. This provides evidence that the development of an OPA model could be utilised as a much needed unique large animal translational model for human lung cancer research.

What outputs do you think you will see at the end of this project?

Our plan to inoculate sheep with the tumour causing virus (Jaagsiekte sheep retrovirus) will allow us to gather significant new information about OPA disease progression and transmission. These results will inform “best practice” for the control of OPA and will form the basis of new advice for farmers and vets. We will publish our results in high impact peer-reviewed scientific journals and generate a policy document for dissemination to UK and devolved governmental bodies. Talks will be presented at national events such as Sheep Veterinary Society and Sheep Breeders Round Table and also at educational events arranged by relevant UK-wide bodies. We have the benefit of the local Institute's public engagement communications teams who will support all our impact activities through advice, press releases and social media dissemination of each event. Additional outputs will include the generation of an archive of biological samples from the point of viral infection to the development of OPA tumours. These sample can be used for future experiments such as the development of OPA diagnostic tests.

Our project will also result in the generation of an OPA model that can also be used for translational human lung cancer research studies. These results will be published in high impact peer-reviewed oncology journals. Once the model is established and the initial objectives relating to OPA disease control are addressed, we will perform initial proof-of-concept experiments to demonstrate that OPA tumours can be used in human lung cancer translational studies. These experiments will involve obtaining preliminary data on how injections can be safely made into OPA tumours which will inform the development of novel methods for the localised treatment of human lung cancer.

Who or what will benefit from these outputs, and how?

Fundamental to our project is the development of OPA control programmes that will benefit farmers economically and improve sheep health and welfare. OPA is a debilitating disease that has significant animal welfare issues, and any project that results in the reduction of OPA incidence and those that can lead to OPA-free flocks will ultimately improve animal welfare standards and support sustainable farming and food security not just in the UK but in any sheep breeding country.

The results from our work will inform “best practice” for OPA control in the future and will form the basis of new advice for farmers and vets. Furthermore, we will generate a policy document for dissemination to UK and devolved governmental bodies. Workshops will be arranged to which we will invite the main stakeholders including vets, sheep society representatives, industry leading farmers and government policymakers to participate in knowledge exchange to help refine our final OPA control advice including informing the potential for an OPA assurance scheme. Thereafter the policy document and documents for dissemination of the new advice to stakeholders will be prepared. We aim to distribute these findings to all 6,600 members of the National Sheep Association and will work at public events such as sheep health seminars, agricultural shows and National Sheep Association events to increase awareness of our findings. In addition, the information will be shared in the farming and veterinary press, and using outreach activities such as webinars and public meetings.

Our results from the development of a robust experimental induced OPA model has the significant potential to improve human lung cancer research. Human lung cancer research relies heavily on in vitro cell line and small animal models. Unfortunately overall 5-year survival rates remain at only 15%.

Comparative translational models of human lung cancer, such as a reproducible experimentally induced OPA model has the potential for improving the understanding of some aspects of human lung cancers and in developing new treatments.

Our first translational experiments will be conducted in terminally anaesthetised sheep and will indicate the likely translational relevance of our model in terms of future human lung cancer studies. This initial study will evaluate if intra-tumoural injections can be performed safely in this model. It will also provide information regarding the tissue distribution and retention of injected material. This information is essential for progressing the model into subsequent recovery studies.

How will you look to maximise the outputs of this work?

This project is multidisciplinary and involves colleagues from a number of scientific institutes.
The Institute primarily involved in this research programme is a world class research institute with a track record of delivering cutting-edge research. It is the UK's foremost centre for livestock genetics and genomics and a world leader in methodologies for analysing animal and pathogen genomes. The Institute undertakes basic and translational science to tackle pressing issues in animal health and welfare, their implications for human health and the role of animals in the food chain. This project will also make use of large animal research and imaging facility which is fully supported and maintained by dedicated animal services and expert technical personnel.

Our collaborating institute is recognised internationally as a research leader in livestock infectious diseases. Research focuses on endemic pathogens that impact the health,

welfare and productivity of livestock, zoonotic and food-borne pathogens that affect human health and pathogens relevant to comparative medicine. Outputs include disease control programmes, diagnostic tools and novel vaccine approaches. This institute has links with Pfizer Global Alliances, UK Universities and other relevant institutions. This institute has been the grant holder for various projects funded through the BBSRC, Wellcome Trust, MRC, EU, NIH and others. Commercialisation of research includes patents, licence agreements, spin-out companies and research collaborations.

Local statistical expertise will be utilised to develop and use computational and mathematical methods in statistics, process-based modelling and bioinformatics. This resource is recognised internationally for work at the interface between mathematics and the agricultural and environmental sciences with a specific interest in livestock and wildlife disease. This work includes statistical analysis of challenge experiments and field observations, diagnostic test evaluation and epidemiological modelling. This institute has been at the forefront of statistical inference methods for dynamic epidemic models.

A commercial company involved in the research and development of medical devices and pharmaceuticals has provided funding for this project. This funding will drive the translational aspects of the model forward.

Species and numbers of animals expected to be used

- Sheep: 55

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All animal experimentation raises ethical concerns. The experiments proposed here will be conducted in the natural target species and have the ultimate aim to prevent disease in that species. Ovine pulmonary adenocarcinoma causes significant animal suffering and we believe that efforts should be made to reduce the number of animals affected and minimise the severity of suffering. The use of 48 weaned lambs in this experiment will provide a great deal of information that will be valuable not only for controlling the disease but also for advancing human lung cancer research.

OPA is endemic in the UK and the use of naturally-occurring cases as opposed to experimentally induced disease for research is a well-recognised technique. However, the use of naturally-occurring diseased animals for veterinary and translational studies has significant limitations. Firstly, transport of affected animals from the farm of origin to the research facility has ethical and welfare implications.
Even diseased animals which are identified on farm through thoracic ultrasound which are asymptomatic can unfortunately develop clinical signs arising from the stresses of transport. Secondary infections related to transport can also cause asymptomatic animals to develop clinical signs very quickly. The second major limiting factor for the use of naturally-occurring diseased animals in our research study is that OPA affected animals tend to be diagnosed at a relatively late stage of the disease process, typically either when tumours are greater than 1 cm based on ultrasound exam or when tumours are large enough to compromise respiratory function. By this stage the animals have significant

pathology and cannot be used to investigate early stage disease.

These issues can be overcome by the use of the experimentally induced OPA model using lambs and provides justification for our use of the model. The lambs will be transported to the research facility before viral infection and this directly overcomes any welfare issues related to transport of infected animals. The experimental OPA model also allows us to monitor JSRV emitted from sheep from time of infection through to the development of OPA tumours. The stage of infection/disease progression at which sheep become infectious and are thus a transmission risk to their flock-mates is currently unknown, our study aims to address this issue. This situation is only possible using the experimental infection model and is the main reason why other models cannot be used to address our aims and hypothesis. Characterisation of tumour characteristics, including number of lesions and growth rates from the time of infection is also required to be able to generate a robust OPA model for translational studies.

2-4 month old weaned lambs have been chosen for use in this study as OPA tumour development from JSRV inoculation has been related to the age at which inoculation is performed. Younger animals are more susceptible to OPA tumour development following inoculation. However the use of week old lambs has husbandry and welfare issues for both the lambs and their mothers. We have therefore chosen to use young but weaned lambs to overcome these issues. Previous experience indicates that following intra-tracheal instillation of virus typically at least 75% of lambs will develop small (1-5mm diameter) OPA lesions within the first few months and all will develop lesions by 9 months. We are therefore confident that a similar number of lambs in this study will develop local lung lesions following bronchoscopic delivery.

Our initial experiments in protocol 1 utilised 4-month-old, weaned lambs which underwent JSRV inoculation. So far, our CT and ultrasound imaging results have indicated that bronchoscopic instillation of wild type JSRV at 1:20 (low) and 1:5 (intermediate) viral doses resulted in localised OPA tumour development in approximately 50% of the animals. Although this is a little lower than our previous 75% tumour uptake rate following intra-tracheal instillation, we currently have not performed post-mortem examinations (PME) on all the animals, and it may be that some of our lambs have microscopic tumour formation that as yet is undetectable by CT or ultrasound. We will need to wait until all animals complete the study and undergo tissue collection at PME for definitive diagnosis and for us to obtain a final percentage of OPA positive cases. Tumours from the wild type JSRV at 1:20 (low) and 1:5 (intermediate) viral doses develop into a size useable for translational studies at approximately 20 weeks post-instillation (objective 5). In order to validate these results and to improve both the uptake and rate of tumour development we wish to repeat protocol 1 using younger weaned animals (2-3 month of age) instilled with either low (9 animals) or intermediate (9 animals) viral doses. We base these modifications on the hypothesis that younger animals should have improved tumour uptake rates as the cell type that JSRV infects (proliferating type II pneumocytes) is more prevalent in younger animals. Nine animals in each of the low and intermediate viral dose groups will allow us to compare tumour uptake and growth rates in these younger animals with our original experimental group results. These additional animals will also provide further data to address our research question regarding JSRV shedding in relation to OPA tumour development and progression (objectives 1, 2 and 6). This will be important for developing models of within-flock JSRV transmission and generation of new advice for OPA control (objectives 3 and 4).

Currently only 1 lamb instilled with replicant defective-JSRV (RD-JSRV) has showed CT

imaging findings suggestive of OPA tumour development. Before pursuing further in vivo experiments using this virus we wish to wait until all animals complete the study and undergo tissue collection at PME for definitive diagnosis and for us to obtain a final percentage of OPA positive cases. So far, no lambs instilled with the highest dose of wild type JSRV have developed tumours based on their imaging results. It may have been that this dose caused a significant immune reaction that enable the animals to clear the virus. This will be investigated though PME and collected tissue samples.

Typically, what will be done to an animal used in your project?

The majority of the regulated procedures will be conducted on anaesthesied animals. These procedures will include bronchoscopic virus instillation, CT imaging, ultrasound and sample collection (blood, nasal and breath condensates). During the initial anaesthetic procedure lambs will undergo CT imaging, ultrasound and sample collection followed by bronchoscopic virus instillation. We anticipated this will be the longest procedure the lambs will undergo but should last no longer than 1 hour. Animals will be recovered from anaesthesia and be housed in groups according to their treatment. Animals will subsequently undergo anaesthesia, CT imaging, ultrasound and sample collection at 4 weekly intervals for up to a maximum of 9 months following bronchoscopy. Conscious thoracic ultrasound may also be performed on lambs every 2 weeks after anaesthesia. Animals will be taken through a race to undergo thoracic ultrasound, this procedure should only take 2-4 minutes per sheep.

Anaesthesia will be undertaken only by specialist veterinary anaesthetists who will ensure proper induction and maintenance of anaesthesia suitable for our study. Animals will typically receive an intra- muscular or intra-venous sedative injection prior to induction of anaesthesia. This will act to sedate the lambs and act and reduce stress levels. Only following the induction of general anaesthesia will the procedures be performed. Conducting these sampling procedures under anaesthesia will reduce their potential to cause mild adverse effects on the sheep - as these effects are likely to be transient and will not last beyond the anaesthetic procedure itself.

Following virus instillation animals will undergo daily observation and clinical examination as required. If any animals develop clinical signs associated with OPA disease progression that do not respond to treatment they will be killed. All animals will be killed by a schedule 1 method. The final CT imaging and sample collection procedure conducted on all sheep will be formed as a non-recovery procedure with the animal killed without allowing it to regain consciousness.

Up to 15 subclinical animals that develop moderately sized (approximately 5-10 cm), localised (peripheral or airway invading) OPA tumours (based on CT and ultrasound imaging) will undergo evaluation of intra-tumoral injections during the last anaesthesia event. These animals will be killed without allowing them to regain consciousness.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect all the animals undergoing anaesthesia, sample collection and virus/media bronchoscopic delivery will only experience mild severity. CT scanning, ultrasound, blood sampling, breath condensate collection, nasal swabbing and bronchoscopy are considered to be a safe and well tolerated procedures. Conducting CT, ultrasound, bronchoscopy and sampling procedures under anaesthesia will reduce their potential to cause mild adverse

effects on the sheep - as these effects are likely to be transient and will not last beyond the anaesthetic procedure itself. Pre-emptive analgesia will also be provided. Conscious thoracic ultrasound is a non-invasive procedure that should only take 2-4 minutes per sheep.

Virus instilled animals have the potential to develop clinical signs of OPA disease progression over the time frame of the experiment. Clinical signs of progressive OPA disease include: weight loss, increased respiratory rate and effort, cough and fluid draining from the nostrils. The risks of lambs developing these signs of progressive disease will be mitigated with daily monitoring of the animals with clinical examinations. Where an independent member of the animal care and welfare team suspects clinical abnormality on the basis of reference to animal record sheets an immediate consultation with the named veterinary surgeon (NVS) will be implemented. If treatment is necessary and fails to improve/resolve the issue within a period specified by the NVS, the animal will be euthanised by a schedule 1 method followed by post-mortem examination, including the collection of multiple lung samples for histological examination in order to confirm the nature and extent of OPA.

Intra-tracheal instillation of JSRV results in tumour foci developing throughout the lungs. This disease presentation can increase the risk of animals requiring euthanasia due to the potentially rapid development of clinical signs and produces a model which is not representative of primary human lung cancer. Bronchoscopic instillation of JSRV or replication-defective JSRV (RDI-JSRV) to specifically localise its delivery within the lungs of lambs may overcome these limitations of the current model and reduce the risk of adverse affects occurring.

From our initial experiments we have found that sheep which develop large, localised lesions within 24 weeks of localised bronchoscopic virus instillation maintained a normal appetite and continued to gain weight. During this time, none of the animals showed clinical sign of disease and were unremarkable on clinical examination immediately before euthanasia for the termination of the study. Although these affected animals have the potential to develop clinical signs, our results so far indicate that the procedures and monitoring protocols are mitigating the risks.

Expected severity categories and the 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 all the animals undergoing anaesthesia, sample collection and virus/media bronchoscopic delivery will only experience mild severity. CT scanning, ultrasound, blood sampling, breath condensate sampling, nasal swabbing and bronchoscopy are considered to be a safe and well tolerated procedures. As CT, ultrasound, bronchoscopy and sample collection are all completed under general anaesthesia the potential adverse effects from the procedures will not last for the duration of anaesthesia. However, a moderate severity category has been deemed necessary as virus instilled animals have the potential to develop clinical signs of OPA disease progression over the time frame of the experiment. Animals will also undergo repeated anaesthetic procedures for sample collection, ultrasound and CT imaging. Although these are mild severity categories, performing these procedures multiple times would warrant a moderate category.

Conscious thoracic ultrasound, performed on lambs every 2 weeks after anaesthesia, is considered to be a safe and well tolerated procedure. Animals will be taken through a race, with the ultrasound procedure only taking 2-4 minutes per sheep.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have 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 proposed here will be conducted in the natural target species and have the ultimate aim to prevent disease in that species. It is necessary to use animals in this project in order to meet the primary objectives because there are no alternative systems that offer the facility to assess OPA disease progression right from the time of viral infection. The experimentally induced OPA model is the only way of obtaining data from the moment of virus inoculation.

Currently there is no large animal lung cancer model that can be used for translational studies. This developed model will be unique and essential to improve lung cancer research. The use of in vitro cell line studies and mouse models are unable to be successfully utilised for studies that require advanced imaging, ultrasound, bronchoscopy or surgery.

Which non-animal alternatives did you consider for use in this project?

The use of ex vivo lung tissue culture techniques enables us to do many in vitro experiments looking at initial infection and transformation that could previously only be done in vivo.

The use of OPA cell lines is appropriate for some studies such as proteomic and genomic techniques to elucidate the transformation process.

Why were they not suitable?

In vitro OPA cell line experiments are hampered by the lack of a cell line that can fully support JSRV replication. This issue has limited the amount of in vitro OPA research that has been performed. Some studies have therefore focused on the use of primary OPA tumour cells; however, extended in vitro culture of these cells typically leads to a cessation in virus production. Lung tissue explants can overcome some of the issues related to in vitro cell line experiments, however culture can only be maintained for relatively short periods of time. In vitro work either with cell lines or tissue explants can not account for the animals immune response and tissue microenvironment which are likely essential factors involved in JSRV infection and tumour development in the live animal.

Naturally-occurring JSRV infection with subsequent OPA disease progression is thought to be a slowly progressing disease that can take months to years to cause clinical disease. These time frames cannot be simulated in a laboratory setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This study has been carefully considered with input from expert statistical advice in order to use the minimum number of animals to provide useful results. Approval by the local Experiments and Ethical Review Committee is only given where the animal numbers have been demonstrated to be the minimum consistent with deriving statistically-significant results and where statistical advice has been demonstrated.

18 lambs will undergo JSRV inoculation and 6 will undergo RD-JSRV inoculation (virally treated groups) and 6 will undergo sham inoculation (control group). The use of 30 weaned lambs in this experiment will provide a great deal of information that will be valuable for controlling the disease and developing the translational model. The addition of a further 18 lambs for a second round of experiments will use the most promising viral doses and validate the initial results. Nine animals in each of the additional low and intermediate viral dose groups will allow us to compare tumour uptake and growth rates with our original experimental group results. We have included 7 additional lambs to mitigate for removal of lambs from the study at the first CT scan due to pre-existing lung pathology.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

One of our objectives is to determine the infectivity of JSRV obtained from different biological samples. Undertaking this study in vivo would require a very large number of sheep over an extended period of time. Therefore, we will use two in vitro approaches for measuring JSRV infectivity in breath condensates and nasal swabs. First, we will measure infectivity using a cell line assay. Although there are no cell lines available that are fully permissive for JSRV replication; the CRFK-ovH2 cell line allows JSRV entry and integration and this can be used to quantify infectivity. We will expose limiting dilutions of the samples to CRFK-ovH2 cells and 4 days later infection will be detected by PCR of DNA extracted from the infected cells. Secondly, we will infect ex vivo precision cut lung slices with JSRV. Limiting dilutions of JSRV or sample will be added to lung slices to establish infectious dose. The read out will be immunohistochemistry of lung slices and RT-qPCR of culture supernatant at 14 days post-infection.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each study is carefully considered with local expert statistical advice in order to use the minimum number of animals possible to give useful results. This will also involve generating a model of within- flock JSRV transmission which can be used to assess different interventions aimed at control of OPA. This model is based upon the results obtained from the in vivo study.

This project is designed to generate novel essential results required to generate actionable OPA control measures. To enable us to achieve our aims we are utilising methods which our group is extremely knowledgeable in and have been previously validated in our labs. We will use these methods to generate novel results which have previously never been reported in the literature. The methods we shall use include:

Generation of JSRV21 in vitro by transient transfection of cells with pCMV2-JS21
Generation of RD-JSRV
Bronchoscopic instillation of JSRV21 or RD-JSRV into a specific lung lobe of anaesthesied lambs

Thoracic CT and ultrasound imaging to monitor tumour development with collection of blood, deep nasal swab and breath condensate samples

The concentration of JSRV in each breath condensate and nasal swab sample will be estimated as copy number/volume by RT-qPCR

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using the JSRV experimentally induced OPA model in 2-4 month old lambs.

The majority of the regulated procedures will be conducted on anaesthesied animals. Conducting bronchoscopic virus instillation, ultrasound, CT imaging and sample collection (blood, nasal and breath condensates) under anaesthesia will reduce their potential to cause mild adverse effects on the sheep - as these effects are likely to be transient and will not last beyond the anaesthetic procedure itself. The final CT imaging and sample collection procedure conducted on all sheep will be formed as a non- recovery procedure with the animal killed without allowing it to regain consciousness. Conscious thoracic ultrasound is non-invasive and unlikely to cause any adverse effects.

The use of the experimentally induced OPA model is also considered a refinement over using naturally- occurring diseased animals. Transport of naturally-occurring OPA diseased sheep from the farm of origin to the research facility has potential ethical and welfare implications. Even diseased animals which are identified on farm through thoracic ultrasound which are asymptomatic can unfortunately develop clinical signs arising from the stresses of transport. Secondary infections related to transport can also cause asymptomatic animals to develop clinical signs very quickly. The use of the experimentally induced OPA model means that these potential welfare issues associated with transporting naturally affected OPA cases are overcome.

Why can’t you use animals that are less sentient?

Sheep are the only model we can use for experimental infection as JSRV cannot infect any other species except for goats. The experiments proposed here will be conducted in the natural target species and have the ultimate aim to prevent disease in that species. Performing experiments on sheep will therefore provide the most comprehensive data directly related to that species. There is also no other naturally-occurring or inducible large animal lung cancer model for human translational studies.

Although lambs will undergo multiple recovery procedures over the 9 month time frame, the final CT imaging and sample collection procedure conducted on all sheep will be formed as a non-recovery procedure with the animal killed without allowing it to regain consciousness.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We aim to minimise any stress or discomfort to experimental animals. Sheep will be housed in groups of more than 2 with respect to their treatment i.e. virus or sham inoculated. Control animals will be housed separately from virus treated animals to reduce the possibility of cross infection. Control animals will undergo anaesthesia, CT imaging, ultrasound and sampling prior to virus instilled animals, with strict cleaning and disinfection procedures after each animal. Again these measures will aim to reduce the possibility of cross infection.

Animals with any signs of distress or disease will be examined by a vet and treated as appropriate or euthanised. Humane endpoints will be based on moderate severity limits, with consideration of weight loss, body condition, appetite, respiration, behaviour and dehydration according to a clinical scoring system.

From our initial experiments we have found that sheep which develop large, localised lesions within 24 weeks of localised bronchoscopic virus instillation maintained a normal appetite and continued to gain weight. During this time, none of the animals showed clinical sign of disease and were unremarkable on clinical examination immediately before euthanasia for the termination of the study. Although these affected animals have the potential to develop clinical signs our results so far indicate that the procedures and monitoring protocols are mitigating the risks.

In the case of induction of experimental OPA, a recent refinement is that we can check the infectivity of the viral inoculum by infection of precision cut lung slice cultures before moving to in vivo studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have used the PREPARE guidelines for planning the animal experiments. To enable us to achieve our aims we are utilising methods which our group is extremely knowledgeable in and have been previously validated in our labs. We will use these methods to generate novel results which have previously never been reported in the literature. The methods we shall use and have previously published upon include:

Generation of JSRV21 in vitro by transient transfection of cells with pCMV2-JS21
Generation of RD-JSRV

Bronchoscopic instillation of JSRV into a specific lung lobe of anaesthesied lambs
Thoracic CT and ultrasound imaging to monitor tumour development with collection of blood, deep nasal swab and breath condensate samples

The concentration of JSRV in each breath condensate and nasal swab sample will be estimated as copy number/volume by RT-qPCR

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All group members will regularly keep updated about 3R advances largely through online courses and research.

Online resources will include: Pubmed and Google searches, Altweb (global clearinghouse for information on the 3Rs; http://altweb.jhsph.edu/resources/links.html), Norecopa (https://norecopa.no/3r- guide-database), National Centre for the 3Rs (www.nc3rs.org.uk/informationportal), Alt.Tox (advancing non-animal methods of toxicity testing through online discussion and information exchange; http://alttox.org), AnimAlt-ZEBET (https://www.bfr.bund.de/en/zebet_database_on_alternatives_to_animal_experiments_on_the_internet_____animalt_zebet_-1508.html), DB-ALM ECVAM Database Service on Alternative Methods to Animal Experimentation (https://ecvam-dbalm.jrc.ec.europa.eu/), Fund for the Replacement of Animals in Medical Experiments (http://www.frame.org.uk)

Regular contact with the veterinary services at our Institutes will provide first contact for any 3R updates. Local courses run through these institutes will also be attended.

Home Office

208. Diagnosis of Toxoplasmosis

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

Toxoplasma, Toxoplasmosis, Diagnosis

Animal types	Life stages
Mice	adult
Chicken	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 produce live *Toxoplasma gondii* cells essential for use in highly specialised laboratory tests for the diagnosis of toxoplasma infection in patients who may have severe or life- threatening disease. These include patients who are immunosuppressed (tissue/organ transplant recipients, patients receiving cancer therapies, and HIV) and during pregnancy, where the unborn child may be at risk of severe congenital toxoplasma infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Rapid and accurate diagnosis of toxoplasmosis can support best treatment of patients, thereby reducing severity of disease. The tests in which the toxoplasma cells are used, measure the ability of the patient's antibodies to kill them. For each patient blood sample tested, toxoplasma cells are added at a range of different dilutions of the patient's blood, in order to precisely determine the level of immunity to toxoplasma. The levels of antibody

measured in this way, and comparing levels of antibody in blood samples taken from a patient over a period of time, are critical for clinicians in assessing whether or not a patient has acute or active infection. Diagnosis of active infection is important in patients with compromised immunity, as toxoplasma can reactivate in these patients to progress rapidly to severe or life-threatening disease. This capability to rapidly diagnose and quickly treat reactivated infection is critical, as there is no effective antimicrobial treatment against the latent/quiescent form of the parasite that can reduce risk of reactivation.

These specialist toxoplasma tests are also used to measure toxoplasma antibody levels in specimens sent out to hospital laboratories across the NHS and internationally, through an accredited quality assurance scheme to help them calibrate their first-line toxoplasma tests and ensure these are performing accurately.

What outputs do you think you will see at the end of this project?

The project will allow continued delivery of:

Specialist diagnostic testing to patients in England and Wales

Monitoring of patterns of toxoplasmosis in the population (epidemiological surveillance)

Providing support to hospital laboratories in ensuring their local testing is accurate and of high quality

Supporting the development and evaluation of more accurate diagnostic tests for toxoplasmosis

Underpinning collaborations with international partners to develop and agree guidelines for best practice in the diagnosis and management of severe or life-threatening disease in vulnerable clinical groups.

Who or what will benefit from these outputs, and how?

The rapid delivery of reference tests for toxoplasma will support clinical intervention aimed at a reduction in morbidity and mortality in the human population associated with toxoplasma infection. While toxoplasmosis in the immunocompetent typically presents as a mild to moderate ‘flu-like of glandular fever-like illness usually requiring no more medical intervention, infection in the unborn child, HIV/AIDS patients and other immunosuppressed patients (organ transplant recipients, cancer patients, etc.) can cause severe disease, or be life-threatening or, in unborn children that survive to full term, can result in severely impaired vision or blindness, and damage to the CNS that can affect child development and CNS function later in life. Delivery of these critical health gains is currently being provided in an ongoing manner through the existing project licence, and the current application for renewal is so that these can continue to be delivered.
In the immunocompetent, where toxoplasma infection is usually self-limiting, confirmation of toxoplasma infection excludes differential diagnoses such as lymphoma so that infected individuals can be reassured and also will not need to undergo further investigations. Again, continuation of this service in the new licence application will ensure these services can be maintained and the population can continue to benefit accordingly.

Approximately 12-14,000 clinical specimens per annum from the NHS require specialist testing to support provision of healthcare services within the UK, with an additional 200-

500 specimens per annum received from healthcare services in Ireland.

Testing is also carried out for all candidate specimens that will be included in an international quality assurance scheme. Continuation of this support for will also be assured through the new licence application.

Support is also provided for national surveillance and risk assessment of toxoplasma infection in the UK population through submission of test results to an enhanced surveillance scheme. The findings are published regularly in national infection data. The results both inform assessment of burden of disease, and provide insight into the relative contribution of food-borne toxoplasmosis, and toxoplasma infection acquired from environmental sources.

The work undertaken also contributes to international initiatives, for example, aimed at developing standardised guidelines for best-practice in the screening, risk assessment and diagnosis of clinically-vulnerable patients, e.g. bone marrow transplant recipients. This project (Diagnosis of Toxoplasmosis) provides the vital components of the toxoplasma testing that underpin these international collaborations.

How will you look to maximise the outputs of this work?

The specialist testing undertaken with the toxoplasma cells produced, is fully-funded by the NHS and offered as part of an accredited diagnostic service.

Data from test results are anonymised and provided quarterly for publication as part of the UK national disease surveillance programme.

The performance of the supported quality assurance scheme for human diagnostic laboratory testing is published periodically.

Clinical case reports are published in the medical and scientific literature

All other findings of clinical and scientific interest are published in relevant peer-reviewed journals.

Species and numbers of animals expected to be used

- Mice: 1500 per year for 5 years (7500 total)
- Domestic fowl: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Toxoplasma gondii is a single-celled parasitic organism that must live inside other cells as part of its life cycle. Thus, conventional production methods using *in vitro* growth media, for example, are not possible. While it is possible to grow toxoplasma in one specific type of in vitro medium (in living cells grown in tissue cell culture), this method has been extensively investigated and toxoplasma produced this way have been confirmed as unsuitable for use in diagnostic testing of patients (due partly to having a lower viability than those grown *in vivo)*. This is important because one of the key specialist toxoplasma tests offered,

involves measuring the impact of the patient's antibodies on viability of toxoplasma cells.

Adult mice provide suitable numbers of fully viable toxoplasma cells to meet diagnostic testing demand. Culture of toxoplasma in fertile chicken eggs provides toxoplasma antigens in a robust state suitable for inclusion in enzyme immunoassay-based diagnostic tests for detection of antibodies against toxoplasma.

Typically, what will be done to an animal used in your project?

Mice are injected with a measured number of toxoplasma cells and are then returned to their cage. After 3 days the mice are killed humanely and toxoplasma cells are harvested. 3 days is chosen as this provides sufficient numbers of toxoplasma cells for testing all patients' specimens while at the same time preventing development in the mice of any severe symptoms.

12-day fertile eggs have a small portion of the shell and outer membrane removed and a measured number of toxoplasma cells are added. The eggs are incubated for 7 days and the embryos are killed by refrigeration at 4°C for more than 4 hours.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection into mice is intraperitoneal (injection through abdominal wall). Expected adverse effects are minor discomfort in less than 1% of administrations during the procedure. This is based on observation during the procedure for signs of discomfort, including increased vocalisation or attempted movement, and post-procedure, looking for signs of severe lethargy, twitching, trembling, self-imposed isolation from other animals in cage or abnormal posture. Symptomatic infection occurs in less than 0.1% of mice and may include severe lethargy, twitching, trembling, self-imposed isolation from other animals in cage or abnormal posture.

Adverse effects in inoculation of fertile eggs occurs in less than 0.2% of administrations and results from damage during removal of the small portion of shell. This presents as damage to shell outside of the area being removed or damage to the inner shell membrane which may result in bleeding during or after inoculation.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity level for inoculation of mice and fertile eggs is moderate in 99.9% and 99.8%, respectively. In the 0.1% (mice) and 0.2% (fertile eggs) showing unexpected adverse effects, animals are humanely killed immediately.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Toxoplasma cells are parasites that grow inside animal hosts cells. Toxoplasma grows less well in tissue culture, which is not suitable to provide the necessary accurate diagnosis of patients that underpins potentially life-saving treatment. This is because the toxoplasma cells produced in this way are less viable and more morphologically heterogeneous (typically smaller and more variable in shape and size) than cells produced by *in vivo* culture. This is a major challenge, as the specialist diagnostic test (Sabin-Feldman Dye Test) in which the toxoplasma cells are used, is a method requiring examination of the toxoplasma cells by microscopy to measure the level of toxoplasma cell killing elicited by the patient's antibodies. Use of the less viable and smaller toxoplasma cells derived from tissue culture renders both overall diagnosis, and quantitative measures of immunity, less accurate and more unreliable.

Which non-animal alternatives did you consider for use in this project?

Extensive developmental work and assessment of toxoplasma cells grown in tissue culture has been undertaken, as this would be a highly desirable alternative method for their production. Toxoplasma cells produced in this way are less viable and more morphologically heterogeneous (typically smaller and more variable in shape and size) than cells produced by in vivo culture.

Why were they not suitable?

Evaluation of cells produced this way, when incorporated into the human diagnostic test, have produced inconsistent and unreliable results. This includes a significant number of false positive and false negative results, as well as inconsistencies in the levels of antibody measured. Each of these errors can lead to errors in patient management that may have severe or life-threatening implications.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on previous years demand for testing. Rates of human toxoplasmosis remain approximately constant, and no change in numbers of tests is therefore anticipated.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Human diagnostic testing has been streamlined to require the minimum number of toxoplasma cells per assay, and frequency of testing within the laboratory has been reduced from 5 days per week to 3 days per week to use the toxoplasma cells more efficiently. In addition, we have both published guidance documents for clinicians who

request toxoplasma testing for their patients in order to minimise the number of test requests we receive, and instituted more detailed review of tests requests that are received, to further reduce numbers. These steps have been to ensure the minimum number of animals is used for growing toxoplasma. Success to date has been a 12% decrease in the number of mice required, while still offering and maintaining a comprehensive national reference service with no additional risk to patient outcome. These measures remain under constant review so that any possible further reduction can be identified as advances in technologies permit.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Numbers of animals have been optimised by balancing frequency of testing (less frequent testing can achieve some reduction in animal numbers) against patient outcome (less frequent testing can delay decisions on patient management). Demand for testing has also been managed through a programme of education to inform requesting doctors as to best use of tests to support patient management, and internal review of test requests to identify specimens not suitable for testing.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are chosen for culture of viable toxoplasma since these are the competent host species for this parasite with the lowest degree of neurological sensitivity where whole tachyzoites can be collected in a form suitable for human diagnostic testing.

Fertile eggs are chosen for culture of toxoplasma since these are the competent host species for this parasite with the lowest degree of neurological sensitivity where toxoplasma antigen can be collected in a form suitable for human diagnostic testing.

Why can’t you use animals that are less sentient?

Mice are chosen for culture of Toxoplasma since these are the competent host species for this parasite with the lowest degree of neurological sensitivity where whole tachyzoites can be collected in a form suitable for human diagnostic testing. Chicken embryos are not suitable for this purpose due to the high levels of contaminating cells and cell debris from the embryo that are present. The test in which chicken embryo-derived antigen is used (Enzyme Immunoassay, EIA) does not require the pure toxoplasma tachyzoite cell suspensions (the small number of murine leukocytes present in mouse- derived toxoplasma cells does not interfere with results) required for the Dye Test assay.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Previous refinements will be maintained both around use of finer hypodermic needles and ensuring these are single-use only, to reduce discomfort during inoculation of mice.

Animals will be housed in an enriched environment for the minimum time and will be monitored regularly when infected so that any early signs of infection can be identified, and action taken to minimise suffering, at the earliest opportunity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance published by the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research, The Norway 3R Centre and National Consensus Platform for the Replacement, Refinement and Reduction of animal experiments (Norecopa), and the Laboratory Animal Science Association.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Current advances and innovation in the 3Rs will be kept under review by monitoring sources such as the newsletter of the National Centre for the Replacement, Refinement and Reduction of Animals in Research, the Laboratory Animal Science Association and the Home Office Liaison, Training and Information Forum. Any initiatives will be discussed with the ELH and NTCO before evaluating and, if successful, introducing in the establishment.

Home Office

209. Mechanisms underlying pathology in diabetes

Project duration

4 years 0 months

Project purpose

- Basic research

Key words

Diabetes, Retina, Therapy, Kidney, Blood vessels

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 enhance our understanding of the molecular mechanisms responsible for harmful changes that occur to the retina in diabetes. These typically manifest as modifications to the retinal blood vessels and we will investigate the role of a novel protein we discovered that is implicated in these changes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The work is important because although there are some treatments available for patients with diabetic eye disease, a substantial number of patients fail to respond to current therapies. The work planned here will shed new light on the causes of diabetic eye disease and directly support work in our laboratory aimed at developing novel therapeutic approaches for these patients.

What outputs do you think you will see at the end of this project?
The primary output will be new information concerning the molecular mechanisms responsible for the development of retinal vascular pathology in diabetic eye disease. The

focus of the work will be leucine-rich alpha-2-glycoprotein 1 (LRG1), a protein that in previous studies we have shown to be induced in various forms of retinal vascular disease. We anticipate that the new insights we obtain will be disseminated primarily in the form of peer-reviewed research publications.

Who or what will benefit from these outputs, and how?

Outputs and research discoveries will be generated and released as we obtain them, hence a realistic expectation of the timescales for such outputs is that we may generate some four or five publications over the course of the work.

How will you look to maximise the outputs of this work?

In parallel to the programme of work set out in this Project Licence, my own lab in collaboration with a colleague, has been developing (with MRC Biomedical Catalyst funding) function-blocking antibodies against LRG1 that are currently being commercialised through a spin-out company. The work in this project will provide key insights and mechanistic information that will significantly strengthen the pre- clinical basic science studies on LRG1 blockade, and hence increase the prospects for the successful advancement of PTx and the longer-term treatment of patients with currently untreatable diabetic eye disease.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse models have proven invaluable in advancing our understanding of the mechanisms underlying pathological changes associated with diabetes. Here we will primarily use two models, namely the Ins2AKITA mouse and the streptozotocin (STZ) mouse. The use of two models significantly strengthens the value of any scientific outputs by obviating potential criticisms that observations may be model or strain-specific. In both models changes to the retina become manifest at an early stage, hence our choice of life stage for experimental work will be in the age range 1-6 months.

Typically, what will be done to an animal used in your project?

For the Ins2AKITA mouse these animals are in-bred as a genetically modified mutant strain, whereas with the STZ mouse animals are injected with STZ after weaning. In experimental work animals may receive intraocular injections under anaesthesia, of various compounds (e.g. function-blocking antibodies against LRG1), and depending on the study these might be single injections, or animals may receive an injection every three or four weeks for three or four months in order to evaluate longer term effects and impacts. We do not anticipate any surgical procedures.

What are the expected impacts and/or adverse effects for the animals during your

project?

We have worked extensively with diabetic mouse models, and have not observed problems with animals experiencing pain, tumours or abnormal behaviour. In general the elevated blood glucose in diabetic mice does not cause adverse effects, though animals do typically drink more and hence produce more urine. This in turn requires that maintenance of these animals demands more frequent changing of bedding and more frequent observation.

Expected severity categories and the 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 levels in the diabetic mice are expected to be moderate, and we would anticipate this to apply to all diabetic animals. Non-diabetic animals used as controls and transgenic animals without a harmful phenotype will also be mild severity. The expected proportions of mild and moderate severity are 50:50.

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?

The complexity of diabetes is something that cannot be replicated in cell culture models or even explants. Only in diabetic animal models can one observe the changes to the retinal vasculature (and indeed other organs such as kidney) typically observed in human patients with diabetes. In particular the consequences of blocking molecules involved in driving the vascular pathology can only be evaluated in animals.

Which non-animal alternatives did you consider for use in this project?

We did consider *in vitro* models that can be used to study the vasculature, generally that involve co- cultures of endothelial cells and pericytes maintained in elevated glucose.

Why were they not suitable?

The in vitro models are unsuitable because they can never replicate the pathological changes observed in the retinas of diabetic mice, that include the growth of microvascular tufts, neoangiogenesis and vessel leakage with retinal swelling.

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For each experiment a protocol will be written following careful consideration of the total number of animals and group sizes required to realistically achieve the objectives based on both relevant publications and previous experience of the model used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use a range of experimental design tools and resources (examples listed below) and consult with our in-house statistician to determine the correct experimental group sizes and design the experiments to minimise the number of experimental groups (e.g. careful consideration of control groups). This will enable the number of animals per experiment to be kept to a minimum, mostly 10-12 animals per group. We will also employ several aids including SyRF and PREPARE (Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing.

Lab Anim. 2018 52:135-141) as this will reduce unnecessary animal usage by ensuring that the appropriate model system is chosen and that an appropriate and robust sample size is employed. We will follow the guidelines set out in the National Centre for the Replacement and Refinement and Reduction of Animals in Research (http://www.nc3rs.org.uk/experimental-design-assistant-eda) and take the following steps to minimize the number of animals used. For wild type mice, we will breed or purchase only as required. Where we breed genetically modified heterozygous mice, surplus WT mice not needed as littermate controls will be used for WT studies. Where required, we will avoid unnecessary production or import of genetically modified mice by searching cryobanks and databases. Examples of resources available include:

NC3R’s mouse database: https://www.nc3rs.org.uk/minimising-use-ga-mice Animal Welfare Management Discussion Group (AWMDG)
Mouse locator: https://mouse-locator.crick.ac.uk PubMed: http://www.ncbi.nlm.nih.gov/
Jackson laboratory: http://jaxmice.jax.org/index.html
Cre transgenic database: http://nagy.mshri.on.ca/cre_new/search/Search.php
What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where needed, the strain used for generating a new colony will be carefully considered to avoid producing unwanted mice. Animals will only be bred if needed, and the breeding programme will be subject to regular review (every two to three months) to optimally meet anticipated demand. Spare animals will be made available to other PPL holders within our Institute for use on other scientific projects. Breeding will be optimised, wherever possible, to produce only the genotype required e.g. homozygous breeding pairs. Cryopreservation of gametes 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.

The mouse models chosen for the investigation of vascular pathology in diabetic eye disease are very well-established models that have been extensively characterised. Using such widely used models allows us to draw on a vast array of additional published data and reduces the need for additional experimental validation. The SyRF on-line facility and the PREPARE guidelines will also assist us in making the correct choice of animal model and by informing us of outcomes from related studies/therapies help refine our experimental protocol. Facilities such as these, and keeping up to date with the literature will also ensure we are alert to new models that may emerge during the course of work, that may have advantages over existing models.

Why can’t you use animals that are less sentient?

Less sentient animals such as Zebra fish, yeast, C. Elegans etc, simply do not have the vascular structures that permit an accurate modelling of human diabetic disease. Similarly embryonic animals or animals under terminal anaesthesia cannot be used because of the duration of elevated blood glucose required for pathological changes to occur.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Diabetic mice typically exhibit minimal changes in welfare, with no reports of pain, and the major management issue being adequate provision of water to meet the increased thirst typically seen in these animals. This in turn necessitates more frequent changing of bedding in cages and closer inspection, something we have managed without problems for many years.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure that experiments are conducted in the most refined way all mice will be monitored regularly to ascertain health status and condition. In all cases animals will be weighed frequently and overall body condition will be used to determine health and establishing endpoints (e.g. see: ILAR Journal
V.41 (2) 2000 Humane Endpoints for Animals Used in Biomedical Research and Testing). Body condition scoring (BCS) will also be used (BCS; Foltz and Ullman-Cullere, 1999. Guidelines for Assessing the Health and Condition of Mice. Lab Animal. 28:28-32). BCS does not preclude other criteria for premature euthanasia (such as hunched posture, ruffled hair coat, reluctance to move), but will be used in conjunction with these standards. At any time the NVS and NACWO will be consulted if there are any questions. BCS scores are determined by frequent visual and hands-on examination of each animal and is particularly helpful in cases where pregnancy, organomegaly, or tumour growth (particularly intra-abdominal growth) may interfere with body weight assessment. This can

be aided by the use of published diagrams (for example see: Ullman-Cullere MH, Foltz CJ. Body condition scoring: a rapid and accurate method for assessing health status in mice. Lab Anim Sci 49:319-323, 1999). Researchers and animal staff will be instructed on specific symptoms, and given instructions on killing criteria.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Institute provides regular bulletins to all PPL holders with information relating to new advances in the 3Rs, and this will be my primary source of new information. Implementation of any new published and validated advances will be undertaken, where necessary through modifications to this Project Licence.

210. Dissecting the intrinsic and extrinsic mechanisms regulating normal and leukemic stem cells

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Blood Stem Cell, Leukaemia, Microenvironment, Bone marrow niche, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study the normal development of blood stem cells and understand how and which alterations might alter this normal development and induce leukaemia. We will also investigate how normal and leukemic cells respond to specific microenvironment factors and investigate whether modulating specific factors could impede the growth of leukaemia. Lastly, we aim to translate our basic laboratory research into clinical benefit.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
Blood stem cell transplantation is a life-saving therapy, employed to reconstitute cancer patients' blood and immune systems. Furthermore, blood stem cell could be engineered to correct genetic diseases or fight infectious agents. Nevertheless, shortage of blood stem cell donors still limits their used.
Understanding what mechanisms regulate blood stem cells self-renewal and their fate decision should provide new tools to generate, and expand blood stem cells and thus exploit their full therapeutic potential. Furthermore, dysregulation of blood stem cells via acquisition of genetic mutations are at the origin of leukemia development. Understanding how leukaemia initiates, propagates and competes with the normal blood compartment will allow us to develop new therapeutic tools.

What outputs do you think you will see at the end of this project?

At the end of the project we anticipate to:

- Have a better understanding of the components of the stem cell niche using visualisation techniques and functional study to elucidate which component of the stroma is/are essential for the maintenance of normal and leukaemic stem cells.

- Unravel how microenvironment cells/factors influence human normal haematopoietic stem cell (HSC) fate decision and thymopoiesis.

- Decipher how pre-malignant and malignant cells outcompete overtime and/or under different stresses (such as inflammation, chemotherapy, radiation, infection, mobilizing agents).

- Provide new insight into the regulation of human haematopoietic stem cell (HSC) during ontogeny and ageing.

We will disseminate our results in conferences and publications as well as in public engagement events.

Who or what will benefit from these outputs, and how?

As we are working directly on human haematopoietic stem cells and leukaemia, data generated should provide:

- new therapeutic avenues to target malignant stem cells while preserving the normal stem cell compartment.

- new ways to expand the stem cell pool providing a new source for cell therapy like BM transplantation.

- new ways to harness human T cells development.

- novel model to investigate the effect of leukaemia in immune cell function and develop new immunotherapy strategies.

How will you look to maximise the outputs of this work?

- Thanks to a constant discussion with the translational team at the Institute, we are able to patent our works, and via direct communications with big pharma and biotechs are in a good position to translate our work.

- Similarly, thanks to good connection with clinicians we can translate some of our work to the clinics.

- Via collaborations (internally and externally), we are and have been able to extend our work (for example: investigating the role of neutrophils in leukemia; developing human thymus).

- Publication of method papers or reviews.

- Participating in public engagement events.

Species and numbers of animals expected to be used

- Mice: 25,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are chosen because they have a short mammalian life span, are easy to maintain, and have similar physiological, anatomical, and genetic traits to humans. They are also the only form of mammal in which transgene technology works well and immunodeficiency models have been established. Furthermore, previous works from our group and others have shown that immunodeficient mice allow human blood system development, both normal and leukaemic. Thus, it is the model of choice to study human normal and leukaemic stem cells.

Indeed, the use of immunodeficient mice has been instrumental in the demonstration of the cancer stem cells in AML as well as to our understanding of the heterogeneity of the normal HSC compartment.

New immunodeficient mice have been used over the years, which provide a better environment for the development of human normal and LSC (like NSG: Il2Rgamma null, $NSG^{W41/W41}$ mice). We will continue to test new models has they become available.

We usually perform our experiment on adult mice, but in some cases, the used of newborn, or juvenile mice to evaluate HSC function during ontogeny might be more appropriate.

Typically, what will be done to an animal used in your project?
In a typical scenario, animals might be subjected to different types of conditioning (irradiation, busulfan etc.), followed by the adoptive transfer of cells via usually intravenous (IV) or intra-bone (IBM) injection. Following the adoptive transfer, mice might also be subjected to imaging under anaesthesia, and they might have blood and/or bone aspiration taken for analysis to test level of engraftment. In some cases, in addition, usually

6 to 12 weeks after adoptive transfer, mice might be subjected to injection of anti- cancer drugs. In other cases, animals might receive different types of challenges (infection, inflammation stress,etc). All mice will finally be killed typically about 12-36 weeks after adoptive transfer, which may involve perfusion or exsanguination under terminal anaesthesia. Lastly, some mice might be implanted with scaffolds either subcutaneously or in limited number of cases, under the kidney capsules. For subcutaneous implantation, an imaging window chamber might be placed to image cells proliferation, and mobility overtime. In all cases, we will use non-invasive technique to follow the development of both normal and leukaemic cells engraftment.

What are the expected impacts and/or adverse effects for the animals during your project?

In most cases, animals may experience pain and weight loss for a short period of time (mild to moderate severity). Mice transplanted with leukaemia might become sick via dissemination of leukemic cells in other tissues. Mice will be monitored daily and at any signs of sickness will be culled. Thus, maximum severity should not exceed a moderate level.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Immuno-compromised mice are usually more susceptible to the procedures and thus might reach the moderate severity limit of the 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?

The gold standard assay to study and prove that the cells studied have normal HSC or leukaemic stem cells activity is to transplant them in vivo into an immuno-deficient mouse model. Thus, the use of animals is currently unavoidable as many facets of stem cell and leukaemia biology are only apparent in the context of the complex in vivo systems in which these cells and diseases naturally occur.
Mice were chosen because they are the only form of mammal in which transgene technology works well and immunodeficiency models have been established. Furthermore, previous work from our group and others have shown that immunodeficient mice allow the development of human blood system both normal and leukaemic. Thus, it is the model of choice to study human normal and leukaemic stem cells (LSCs).

Indeed, the use of immunodeficient mice has been instrumental in the demonstration of the cancer stem cells in AML as well as improving our understanding of the complexity of the

normal HSC compartment We also recently developed a humanised 3D ossicles which allow us to better mimic the human bone marrow niche (Abarrategi et al, JCI,2017).

New transgenic immunodeficient mice are being developed that provide a better environment for the development of human normal and LSC (like NSG: Il2Rgamma null, NSGckit$^{W41/W41}$, NSG-S). We will continue to test new models has they become available.

Which non-animal alternatives did you consider for use in this project?

Over the years, we have developed an ex vivo co-culture system that allows the maintenance of some normal and leukaemic stem cells. We are also in the process of developing a new 3D bioengineered bone marrow system that mimic the human bone marrow niche ex vivo and thus should be provided an even better environment for human normal and leukaemic stem cells.

Why were they not suitable?

Despite continuously improving our ex vivo culture system, the use of animals is currently unavoidable as many facets of stem cell and leukaemia biology are only apparent in the context of the complex in vivo systems in which these cells and diseases naturally occur.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In the design of all our mouse experiments, we strive to use the minimum number of animals that is commensurate with obtaining a robust and reliable result. We will apply optimal experimental designs and statistical analysis as key means of achieving reduction. We will use power calculations to minimise the number of animals used in each experiment. For the design of 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 at least practicable difference between groups of 20%. We will use statistical power analysis under advice from our bioinformatics and statistics core service to determine the appropriate minimum number of animals per study required to gain significant data output.

In general, to investigate the effect of specific over-expression or down-regulation of a target gene, a group of 4 to 6 mice should be enough (control and experimental group). For evaluating the effect of anti-cancer drugs against leukaemia, it is generally more difficult to predict a minimal number based on the heterogeneity in the time to develop leukemia (6 to 18 weeks). Still our past experiences indicate that a group size of around 10-12 mice should be appropriate.

Otherwise, we will use the minimum number of animals to provide an adequate description, generally based on previous experience (our own or from the literature). We

will use a pilot experiment, for example, for preliminary studies, e.g. to optimise the dose of cells, no more than 3 animals per group will be used when possible.

Experiments will be carefully planned to maximise the information obtained per animal and limit the subsequent use of additional animals. For example, organs including bone marrow, blood cells and spleens will be stored and used for multiple experimental purposes. We will also make use of non- invasive imaging to maximise the information obtained per animal.

Collaboration with research colleagues will help to gain maximum data output and reduced redundant breeding/experimentation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Longitudinal measurements, in particular non-invasive ones, such as weight loss, and non-invasive imaging, allow gaining a wealth of information on disease course over time with a minimum number of mice used. Cryopreservation of tissues and cells is routine at the establishment and will ensure that the minimum number of mice is bred and measures are in place to maximise efficiency of breeding schemes with a minimum surplus. Reporting will be based on ARRIVE guidelines. Imaging technology will allow following a cohort of mice over time rather than setting up several experimental groups to allow kinetic analysis, e.g. investigate leukemia invasion overtime or the effect of anti-cancer drugs. We will remain alert to any advances, which will enable the replacement 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?

The efficiency of animal usage is maximised in consultation with colony managers and animal technicians, by careful control of breeding to meet research needs with respect to numbers, phenotypic uniformity and health. This has been greatly facilitated by a mouse database in which every breeding pair and every mouse born are recorded and through which we can readily monitor the numbers of mice we hold.

Where possible, lines will be maintained in a homozygous state, thereby obviating offspring with undesirable genotypes. Littermates genotyped as heterozygous or wild type from the breeding protocol will be used as appropriate age and gender matched controls. This allows optimal use of mouse numbers generated and is the best scientific practice for the study of genetic alterations.

For experiments on development and progression of a leukaemic disease (leukaemogenesis) in response to anti-cancer drugs, we will, whenever possible, make use of bioluminescence imaging to follow a cohort of mice over time, which will substantially reduce the number of mice involved. We will also make used of our new 3D ossicles model, which allows us to implant up to 6 ossicles/mouse, allowing us to reduce the number of mice needed /experiment, as each scaffold could be analysed separately. Lastly, this work program will make optimal use of several tissues, fluids and cell types per individual mouse. This highly integrative approach will maximise the information obtained from the minimum resource.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 because they are the most frequently used mammalian model system to study biology of stem cells and cancer. Other advantages of the mouse model include the availability of antibodies to identify and purify different classes of haematopoietic stem cells and mature blood cells and the availability of in vitro (proliferation, colony-forming cell assays) and in vivo (repopulation experiments and serial transplantations) functional assays.

Previous work from our group and others showed that immunodeficient mice allow human blood system development both normal and leukaemic. This makes the xenotransplantation model a perfect choice to study human normal and leukaemic stem cells.

As most of our work will involve the use of highly immunodeficient mice, we will breed these mice under specific isolators and maintain the experimental mice in IVCs (individually ventilated cages) under a barrier environment, to avoid infections. Furthermore, based on our previous experience working with these mice, when conditioning of the mice is necessary (use of for example sublethal irradation, busulfan etc.) we will be using acidified water and a course of 10 days antibiotic treatment post-conditioning.

In our experiments, we will set clear humane endpoints and, as part of good laboratory practice, write an experimental protocol for each experiment, which will include details of possible adverse effects. All staffs involved in the experiment will have access to these protocols. In addition, when considering which route of administration of substances to employ, we will strive to use the least invasive route while maintaining direct control of dose.

Administration of substances and cells: the route to administer a substance or cells should be such as to achieve “best practice”, that is, to minimize or avoid adverse effects, while minimizing the number of animals used, and maximizing the quality and applicability of results). For that reason, we propose in this project licence, a variety of routes of administration of substances and cells to achieve the scientific objectives, while minimizing the waste of animals' lives. Although in the most cases, we will primarily use standard routes of administration such as intravenous or intraperitoneal injections, the active concentration, volume, stability, and toxicity of a particular substance may require its administration through a non-standard route (like gavage of anti-cancer drugs). Similarly to bypass potential complications arising from failure of the transferred cells to establish in host organs, we will directly inject cells into host organs such as long bones, foetal liver (in case of newborn injection) or implant our scaffold in the kidney capsule (for best vascularisation).

Why can’t you use animals that are less sentient?

Juvenile or adult mice are chosen to study human adult stem cell development as well as leukaemia as they provide at this age an equivalent adult bone marrow microenvironment. Similarly, neonates will be used for studying foetal human hSC development.

Previous work from our group and others showed that juvenile/adult immunodeficient mice allow human blood system development both normal and leukaemic. This makes the xenotransplantation model a perfect choice to study human normal and leukaemic stem cells.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will work closely with the veterinary staff to ensure that we are always refining our protocols to minimize harms for the animals used in our research.

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 the best practice guidelines developed by the National Centre for the Replacement, Refinement, & Reduction of Animals in Research, and the scientific literature for estimation of sample sizes based on power calculations.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep up with the latest developments in the field by reading the relevant literature and we have ongoing discussions of 3R measures in the institute which ensures we are always up to date.

Home Office

211. Dissecting Vertebrate Heart Development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Heart development, Cardiac Defects, Trabeculation, Morphogenesis, Cell biology

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the cellular, molecular and physical mechanisms driving vertebrate heart development and function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

During embryonic development, heart is the first organ to develop and function, as this is crucial for the survival of the embryonic life. As the embryo grows, to support its physiological demands, the developing heart acquires numerous specialized structures to function optimally. Defects in this process of heart development lead to heart disease at birth, a leading cause of morbidity and mortality worldwide. Therefore, in order to understand the causes of heart developmental defects, it is crucial to understand the cellular, molecular and physical mechanisms underlying heart development. The project’s findings will hugely advance our understanding of cardiac defects, thus facilitating the diagnosis and discovery of potential therapies.
What outputs do you think you will see at the end of this project?

The outputs of this work include:

A novel understanding of the underlying cellular processes and physical factors like blood flow that contribute to cardiac trabeculation – a process crucial for heart function.

A better understanding of how genetic factors like Erbb2, Notch and Taz signalling contribute to cardiac trabeculation.

A better understanding of the interplay between genes and mechanical signals regulating heart morphogenesis.

A better understanding of how organs develop inside a growing embryo.

We expect to publish these new findings in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

This project aims to uncover the cellular and molecular events underlying trabecular morphogenesis – a process through which the heart increases its muscle mass for optimal functioning . These findings will be of fundamental importance to the cardiovascular research community, and hugely advance our understanding of cardiac defects, thus facilitating the diagnosis and discovery of potential therapies.
Understanding the interplay between cellular processes and molecular signals is important to reveal the key design factors that regulates the formation of a robust complex organ like the heart. We expect these findings will potentially provide a rationale for tissue-engineering efforts and regenerative medicines.

The tools generated during this project, for example zebrafish transgenic reporters of signalling pathways' activities or transcription factors' expression will be of enormous benefit to the research community as they can be used in numerous other projects where a visual readout of signalling is required. Further, the knowledge generated during this project will inform the wider community of development biology researchers interested in organ development.

How will you look to maximise the outputs of this work?

To maximize the output of the work, we will publish research papers in peer-reviewed journals or preprint servers. We will present our work at both big international meetings and smaller workshops. We will strive to highlight unsuccessful approaches for the benefit of other researchers.

We will collaborate extensively, both internally and externally and this provides another avenue to share details of approaches that were ultimately sub-optimal and how methods were improved.

If we develop new methods, we will publish specific protocols papers.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 95,500

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.

This project aims to understand the cellular, molecular and physical mechanisms driving vertebrate heart development and function. For this we use zebrafish as our model organism. We have chosen this system because of the rapid embryonic development ex utero and optical transparency which allow a direct monitoring and visualization of development, morphology and physiology in the living organism. Also, during the first week of development, zebrafish can survive through passive diffusion of oxygen thus completely bypassing the need of a functional cardiovascular system. This is a unique advantage, as it enables us to use experimental tools compromising cardiac functions while keeping the animal alive, which is otherwise impossible to achieve in other model systems because of early lethality. Thus, zebrafish presents itself as an ideal system to study heart development. Most of our objectives will be achieved by working on zebrafish embryos that are less than 5 days old. To understand the later stages of heart development, we will need to work on juveniles.

Typically, what will be done to an animal used in your project?

We will generate reporter lines expressing biosensors for different signalling pathways or labelling various cellular compartments like the membrane. We will also generate lines in which we have deleted or mutated specific protein components of signalling pathways required for heart development.

These transgenic lines will mainly be studied at the level of embryos and larvae less than 5 days old, and in some cases, we will observe them at the juvenile stages to understand how heart develops and matures.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect any adverse effects for most zebrafish experiments as we are mainly working with transgenic lines that have no negative effect on development or we are monitoring mutant larvae younger than 5 days old. For a small proportion of experiments, when we need to observe the effects of mutations in animals beyond 5 days old, there may be some developmental abnormalities. If we observe abnormal behaviour, such as reduced swimming activity or retreat to dark tank corners, the fish will be killed.

Expected severity categories and the 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 about 10% of fish.

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 heart is the first organ to develop and function during development to sustain the embryonic life. In the most common type of human birth defect (congenital heart diseases), this process of heart development is compromised resulting in structural abnormalities and defective cardiac function. We want to understand the regulatory mechanisms underlying heart development, as we believe this will hugely improve our understanding of cardiac disease and facilitate diagnosis and treatments. We have chosen to work on animals because in order to get a comprehensive and accurate understanding of these regulatory mechanisms, we need to study the heart while it is growing and developing inside an embryo.

Our model system is Zebrafish, which offers several distinct advantages as a powerful vertebrate model organism to study embryonic cardiac development, as explained below:

During the first week of development, zebrafish can survive through passive diffusion thus completely bypassing the need of a functional heart. This allows us to use tools ablating heart functions which is otherwise impossible to achieve in other model systems because of early lethality.

The optically transparent zebrafish embryos allows us to study dynamic developmental events in a living embryo.

In addition to short generation time, zebrafish embryos develop externally which makes them highly accessible to various manipulations.

Recent development of novel genome editing tools (such as the CRISPR/Cas9 system) have made it possible to generate mutant zebrafish lines in a highly efficient manner and the technology is easy to implement.

We will strive to minimize the number of procedures performed on animals deemed to be sentient by doing most of our work on zebrafish embryos and young larvae, before the onset of independent feeding. For some of the experiments, where we need to analyse later stages of heart development, we will need to work on juveniles. We will also use adult or juvenile fish for the fin clipping for genotyping, transient warming to induce expression of transgenes, and gamete harvesting. Concerning the genotyping protocol, mating will be performed as often as possible followed by analysis of phenotype to determine the genotype of the fish as an alternative to fin clipping.

Which non-animal alternatives did you consider for use in this project?

When needed and when suitable, we will strive to complement our in vivo analyses with tissue culture models, organoids and/or mathematical models.

Why were they not suitable?

We can only acquire a limited amount of information about heart development from in vitro systems. The heart's development is inherently linked to its function (heart-beat) and physical cues like blood- flow. These parameters cannot be recapitulated by in vitro systems, thus severely limiting the relevance of the information derived from them.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have calculated the numbers of fish required based on the numbers of mutants and transgenics that we are currently maintaining, plus those that we need to generate to be able to achieve the aims of the project. We are currently maintaining around 40 transgenic lines and 5 mutant lines. In the next five years, we will generate around 25 new transgenic lines and 5 new mutants. We have also analysed each step of the protocols and have estimated the numbers based on the experimental needs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We regularly review our zebrafish stocks and cull any that are no longer required. For all zebrafish lines, we plan to freeze sperm to archive the line. This will allow us to maintain only those lines in the aquarium that we are using in on-going experiments. We will also share our transgenic lines and exchange transgenic lines with other zebrafish labs, in the UK and elsewhere. This will help us to minimise the number of mutant and genetically modified strains that we keep in our own aquarium. Moreover, the stocks of adult mutant fish that we will keep will almost all be heterozygotes carrying recessive mutations and thus phenotypically normal. In addition, because we will share the fish aquarium with a number of other labs, we ensure that multiple workers perform their experiments on the same day to maximise the use of the embryos from a given batch of fish.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We estimate that we will use 400 fish per line over 5 years for maintaining them.

To generate new transgenic lines, the estimated numbers of fish are based on established published protocols, statistics on transgenesis's efficiency from ours and other laboratories. Typically for one transgenic line we will raise 100 F0s, which will be screened by crossing them and subsequently screening their progeny for the transgene. Typically, 20-30 of the adult F0s will be founders. We will choose 2-3 of these and raise their progeny; the others will be culled. For each 'family' we will be raising between 50 and 80 fish which will be genotyped to identify heterozygous carriers of the transgene. The transgenic lines will be then maintained as for other wild type and mutant lines, which corresponds to about 2-4 tanks of around 20 fish each.

We will use a similar strategy to generate mutant fish by CRISPR-Cas9 technology. To produce one mutant line we raise 200-250 founder fish, which are phenotypically normal after microinjection of the CRISPR/Cas9 constructs. To screen for germline transmission and loss-of-function alleles the F0s will be crossed to wild type fish, and their progeny will be screened as embryos for the presence of the mutation. We will pick 2-3 positive founders and raise 200-250 progeny. The others will be culled. The F1s will be screened for the heterozygous mutation by fin clipping. Heterozygous F1s will be further outcrossed to wild type fish to segregate away any non-specific mutations and in-crossed to other transgenic lines (mutant lines to generate double mutants, GFP-transgenic reporter lines). We will raise 100-150 F2s from these crosses and screen them for the desired heterozygous mutations. The mutant line will be then maintained as for other wild type and transgenic lines, which corresponds to about 2-4 tanks of around 20 fish each.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use the zebrafish model for the heart developmental work, as these embryos develop ex utero and can be manipulated genetically and are transparent, and thus ideal for imaging.

We will minimize suffering by paying attention to the fish population's general health, by paying attention to water quality, feeding regimes, and fish population density in each tank. We will check all breeding stock daily and cull any that show signs of significant illness or deformity. Where surgical or other potentially distressing procedures are required, e.g. fin clipping, we will perform them under general anaesthesia with analgesia both pre and post fin clip. Any fish or fish larvae showing signs of distress on recovery from a surgical or other procedure will be killed promptly by an approved method.

Why can’t you use animals that are less sentient?

We use zebrafish embryos for our developmental work, and most of this is on fry less than 5 days old, which are deemed non-sentient and before the onset of independent feeding. To understand the later stages of heart development, we will have to work on juveniles. In this case, we will mostly use non- invasive imaging and where possible, we will imply terminal anesthesia and analgesics

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will achieve most of our objectives using non-invasive microscopic techniques, which does not cause any pain or stress. Surgical procedures like fin clipping will be performed with suitable anaesthesia and animals will be monitored post-surgery to ensure that they recover well. We will also use suitable analgesia for all surgery. Any novel chemical

substance will be tested in a small-scale pilot study for toxicity. We will standardize our imaging experiments on small-scale to first identify the optimal condition for imaging to ensure minimum suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are aware of NC3Rs. We also discuss with colleagues in other research groups new improvements that lead to refinement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay up to date via regularly communication with animal house staff, other scientists in the field and regular visits to the following website https://www.nc3rs.org.uk/3rs-resources.

212. Human antibody generation platform

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Human antibody, Immunotherapy, Immunisation, Ion channels, Membrane proteins

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The objective of this project is to enable the discovery and development of novel fully human antibody therapeutic agents. Immunotherapy has had a significant impact on treating cancer and we believe that antibodies can also have a benefit on treating other therapeutic areas such as inflammation, infection, pain, and neurodegenerative diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

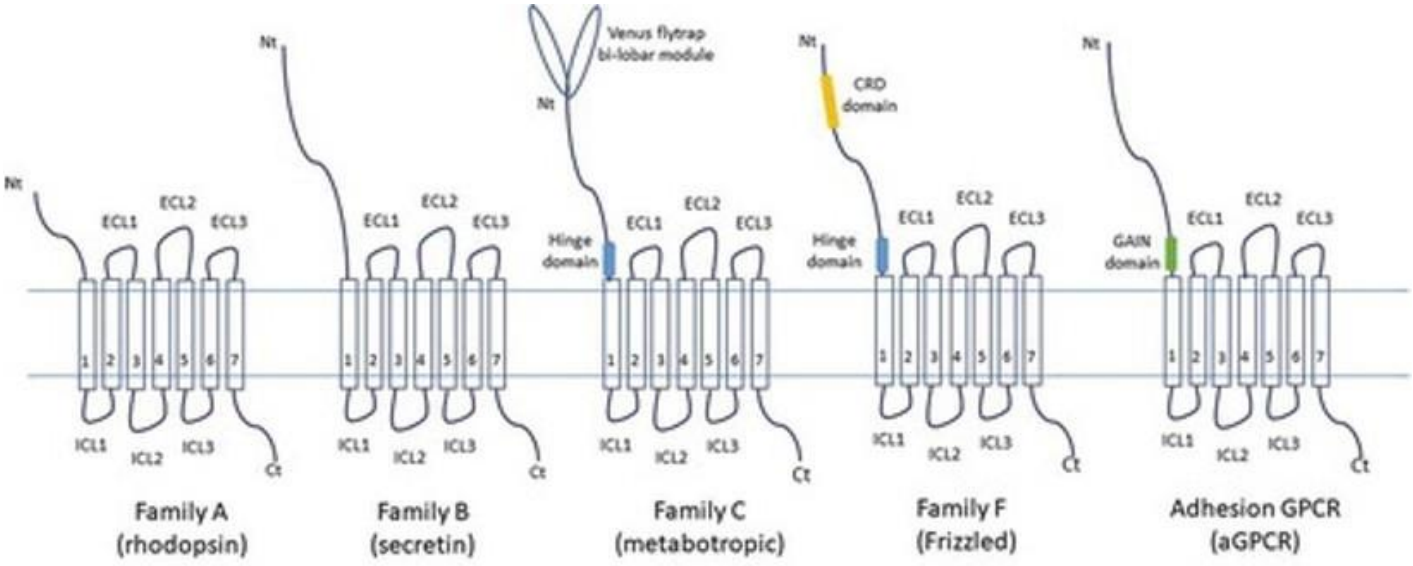
This project will be using mice expressing fully human antibodies which could be used as therapeutics without any further modifications. Because antibodies obtained from these transgenic mice are fully human, they have already been optimised through the immunisation process, and can be used as such as therapeutics in humans.

This discovery technology-driven project will be focussing on diseases involving targets for which no antibody exists because of the structural complexity of the proteins involved which cannot be produced in cell culture. The collaborative approach to generate data for this project will allow us to identify novel biological drugs and to develop new ways using monoclonal antibodies to treat patients with incurable diseases and conditions such as

neurodegenerative diseases, chronic pain, or cancer.

What outputs do you think you will see at the end of this project?

The project will be focussing on targets for which no antibody has been generated yet. For example, Ion channels and G-Protein coupled receptors (GPCR) are protein membranes for which only small molecules have been developed.



These proteins are involved in all therapeutic areas including cancer, inflammation, pain, auto-immune, and neurodegenerative diseases. The output of the project will be to deliver novel fully human therapeutic antibodies with new mechanism of action against this target family. So far only few antibodies have been approved in immunotherapies, such as Mogamulizumab, to treat T-cells leukaemia. Nevertheless, Mogamulizumab is an antibody that was identified from the immunization of mice with a CCR4 peptide exposed on the membrane as the full protein could not be used for immunisation.

This project will obtain antibodies against the whole protein instead of a peptide and there will be no need to make those antibodies human as they did for Mogamulizumab because our mice will produce human antibodies already.

Who or what will benefit from these outputs, and how?

The potential benefits of this project will be the progression of new therapies into clinical development and ultimately onto the market bringing benefit to patients. We intend to develop between two to four antibody candidates for therapies such as cancer, pain, auto-immune diseases, or inflammation into pre-clinical studies over the next 5 years.

How will you look to maximise the outputs of this work?

This is a technology discovery licence, projects are done in collaboration. The project output is fully human antibody. According to published literature and available technology, that is the fastest way to generate a new human therapeutic. However, it is important that each output is fully utilised and developed into the clinic. The partnerability of each asset will be a key criterion when selecting the right target. It is important that we make sure that there is an interest in the field to develop such a drug before we engage in an

immunisation campaign.

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.

Rodents, and especially mice, are very easy to manipulate genetically and as a result have been engineered to produce chimeric mouse/human or fully human antibodies. These mice have been genetically engineered to produce human antibodies after immunisation with an agent which is different enough from a mouse protein to trigger an immune response. Mice will be used as adult with a fully developed immune system.

Typically, what will be done to an animal used in your project?

Because of the project prioritising complex proteins as agent to obtain antibody against, different immunisation protocols have to be used to maximise the chance of success. Standard protein immunisation regime will be used combining purified antigen mixed with an adjuvant to stimulate the immune response. Proteins will also be expressed in mouse cells to keep their biological functions and cells will be directly injected in the mouse abdomen to stimulate immune response. And finally, DNA encoding for the protein of interest will be injected to mice using two different methods (Tail vein delivery and DNA tattooing), those two methods will be performed under general anaesthesia.

Depending on the target type, specific method will be prioritised to reduce the number of animals used.

Over the course of the immunisation, several boosts (up to 6 depending on the method used), will be performed. Antibody levels will be assessed by regular blood sampling. if the antibodies are present and in high enough numbers (high titre), mice will be humanely killed and immune tissues (spleen, lymph nodes, and bone marrow) will be removed. B-cells which are reactive to the protein of interest will be isolated from the immune tissues, mRNA extracted, and genes corresponding to the antibody will be sequenced. No more animals will be used in that experiment.

After a successful antibody discovery campaign, pharmacokinetics (PK) of the human antibody will be assessed by injecting the antibody intravenously to transgenic mice expressing human neonatal Fc receptor by measuring the level of antibody over time by taking a drop of blood sample. This will provide valuable information whether the antibody can be developed as human therapeutics.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice are not expected to have any severe adverse effects from immunisation. Antigen and whole cells immunisations can occasionally lead to mild symptoms such as local

swelling or non-lasting inflammation at injection site. If any adverse effect exceeds the mild category or is not temporary, the animal will be humanely killed. At the end of an immunisation schedule, all mice are humanely killed.

When raising antibodies against proteins expressed using DNA immunisation, special technology has been developed to do so. This technology involves the use of DNA coated gold particles which are introduced to the animal via bombardment of the skin with pressurised gas (Gene gun intradermal injection). This procedure is carried out under general anaesthesia and has minimal associated effects, which can include slight redness of the skin at the site of inoculation.

Expected severity categories and the 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 adjuvants which are very effective in stimulating an immune response can cause tissue reactions in the animals at the site of injection, therefore the use of these adjuvants is carefully controlled, with any reaction being closely monitored. Subsequent blood samples will be taken from an animal in order to test the level of antibodies being produced within the animal. These blood samples will be taken from an appropriate collection site on the animal such as veins/arteries and as such can (but rarely) lead to the formation of bruising and slight skin damage. The level of severity will be mild for most animals.

For the Hydrodynamic tail vein injection, performed under general anaesthesia, 10% animal weight (~2 ml) of a constant concentration of DNA is injected, and the animal is expected to recover within 30 minutes without any side effect. Those two procedures have a moderate severity.

Upon reaching a desired level of circulating antibody against the antigen of interest, an animal will be given terminal anaesthesia, their blood will be collected and antibodies tested. When this has been done, immune tissues (spleen, lymph nodes) will be collected for further scientific use. Although significant adverse signs within any animal used for the production of antibodies are not expected full veterinary attention will be provided should there be any unexpected consequences of any procedure carried out. All animals used for the production of antibodies under the authority of this licence are subject to well defined humane endpoints, which, if experienced, will result in the animal being removed immediately from the study.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The overall aim of the project is to discover new human antibodies towards difficult targets by using specifically engineered strains of mice able to produce human antibodies. The animals used in this licence will be used to develop new antibody therapies to treat conditions involving targets which are difficult to express in solution and for which methods like phage display are not applicable.

Also, ensuring that potential medicines with suitable pharmacokinetic – (what the body does to the drug (PK) properties can be selected for further development to treat human disease effectively. PK is investigated by studying how potential medicines are absorbed and distributed in the body as well as how they are broken down (metabolised) and excreted.

Which non-animal alternatives did you consider for use in this project?

Phage display using fully human universal libraries is the closest method to replace human antibody transgenics animal immunisation and we are using this method currently for projects whose targets can be expressed in solution. This project is mainly focused on therapeutic indications for which recombinant proteins are not stably expressed in solution hence phage panning cannot be performed. Those targets (i.e. Ion channels or G-protein coupled receptors) form an integral part of the membrane and need to be expressed in vivo by using whole cells or DNA immunisation by making animals to produce the target in their cells to trigger an immune response.

Why were they not suitable?

The purpose of using humanised mouse strains to produce human antibodies is that they will use the complete human repertoire of possible antibodies whereas a phage display library will have constraints on the human framework and on the size of the target-recognising loops (CDRs) of the antibody. The complete human repertoire antibody coverage will allow the immunisation process to discover highly potent antibodies which could be ready as therapeutics whereas the potency of antibodies obtained by phage display is limited in potency and will necessitate further lengthy affinity maturation process.

From experience, although the phage panning process is trying to mimic the physiological binding, the conditions the target is attached to a plastic matrix make some portion of the target not visible to the phage presentation and will influence the selection of non-specific binders (i.e. plastic-binder) which will need to be deselected in the process. Some key epitopes could then be masked and antibodies targeting those hidden motifs will be lost. It is therefore important for these targets to be expressed on cells (for cells immunisation) in vitro or in vivo (with DNA immunisation) so they keep their functional structure and trigger specific 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 aim is to run up to 10 projects per year knowing that each immunisation project will use 30 mice (10 mice per protocol) based on the recommendations of the mice developer who has done this for over 10 years. 300 mice will be needed per year and 1500 for the 5 years of the licence for immunisation.
Half of the projects will require knock out generation because the percentage identity between the human target and its mouse equivalent will be over 85%. 20 wild-type females mice and 2 males will be required for this work per project, therefore ~100 per year and 500 for the duration of the licence. We estimated that half of the mice obtained from the breeding will not be required for projects because of male's redundancy (mostly females are required for knock out generation). We will therefore prioritise males for immunisation projects. So, if 2000 mice are required for project work or knock out generation, we estimated that 4000 mice will be required for breeding and project work. Animals not used in projects can used in collaborative research.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For conventional antigen immunisation of standard wild type mice, 5 mice will be enough to generate successful discovery of antibodies. As we will be focussed on difficult targets to be expressed by mouse cells, we will be using three different immunisation methods and increasing the number of mouse per protocol to 10 to be sure that antibody titres will be optimal for at least 5 mice. This recommendation comes from the mouse developer.

Although we will use the mouse developer recommendations for the first few projects, the aim is to select the best protocol for each target and to decrease the number of animals used per protocol if possible. Ideally we should be in a position after several projects to limit the usage of mouse for immunisation but also to select what is the best protocol per target type to increase chance of success with the aim of decreasing the number of mice used per project by half at least.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The first few projects will dictate the success rate per protocol and we will ask the question whether this success could have been obtained with less animals and if an immunisation method is better than the others. We will then implement the result and learnings to subsequent projects so we reach a steady- state whereby we are confident that the least number of animals and methods are used for maximum success.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 strains used in this project have been specifically engineered to produce

human antibodies. Therefore, outputs from this project consist of potential therapeutics ready to enter the clinic without further modifications. Those genetically-altered animals can only be used for this type of approach and are dedicated to human antibody discovery.

Immunisation methods used are mostly mild and will not cause any lasting harm. Antigen and cells immunisation will be performed sub-cutaneously and intra-peritoneally without need of analgesia. DNA immunisations will be performed under general anaesthesia for the ease of the procedure and the safety and wellbeing of the animals.

We will also be incorporating the cutting-edge technology using mRNA encapsulated with LNPs for immunization through subcutaneous, intramuscular or intravenous routes. This technology enables stable and long-term expression of proteins/antigens in vivo from small amount of mRNA, thus shortening the length of immunization experiments. As a result, the duration of immunization experiments is often shorter since the objective (generating humanized antibody) may be met earlier than using conventional protein or DNA-based immunization methods.

Why can’t you use animals that are less sentient?

For immunisation to be successful, we need adult with immune system fully developed. Mice are best organism for this work as they present similar immune response to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experimental procedures may involve a limited number of injections and/or small blood samples (the latter using local analgesic cream) over a period of several weeks. These will be conducted according to best practice guidelines by trained and competent staff. Most procedures will be classed of being of Mild severity and have only a transient impact on the animal. Any concerns regarding the health or welfare of an animal will be discussed with the Named Veterinary Surgeon or the humane killing of the animal. At the end of the procedures, animals will be killed using a recognised humane method detailed in Schedule 1. After every experiment we critically appraise what we do to seek out any ways to improve our models to reduce harm to animals. This strategy has been highly successful and our models continue to show improvement in this area.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs, NORINA, and http://www.procedureswithcare.org.uk/ provide a list, database, and videos of best practices and alternatives to using animals and refinement solutions such as ARRIVE Guidelines and PREPARE (norecopa - https://norecopa.no/prepare)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular interactions with the establishment named persons will allow us to be aware of the latest innovations in the field of the 3Rs but we will also receive regular newsletters from key 3R organisations such as NC3Rs or ALTWEB, and Best practice guidelines on Antibody production.

213. Using delivery platforms for vaccine development against bacterial diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccines, bacteria, antibiotic resistance, Outbreaks, immunology, Vulnerable populations

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 research aims to:

develop and evaluate new vaccines or vaccine components against pathogens affecting humans, for which there is currently no vaccines or vaccines that have insufficient efficacy, and

Investigate and understand the mechanisms underlying successful immune responses to infections, in order to support the generation of novel and more efficient vaccine 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?

Infectious diseases are a leading cause of death and disease worldwide, in particular dramatically affecting vulnerable populations (children and elderly), and low-income

countries. Vaccines represent the best hope for tackling the devastating effects of several dangerous pathogens, because of their cost effectiveness at preventing diseases, as opposed to using diagnostics and treatment, and because of the ability to tackle epidemics, pandemics and outbreaks, as the COVID-19 pandemic has clearly shown Three categories of pathogens are considered in this project:

- Antibiotic resistant bacterial diseases, or diseases contributing to the rise of antibiotic resistance.

- Bacterial diseases that have the potential to cause outbreaks and epidemics

- Pathogens affecting the vulnerable (paediatric and aging populations)

- Therefore, developing new and improved vaccines, that may need fewer doses, be cheaper or can be given needle-free is supporting the goal for healthier lives.

What outputs do you think you will see at the end of this project?

The potential benefits for the 5-year duration of this project are to:

- discover new vaccines or vaccine components against the selected infectious diseases, suitable for the vaccine platforms we are using (Neisseria species, antibiotic-resistance bacteria, bacterial pathogens susceptible to cause outbreaks). We aim to develop or investigate several vaccine candidates for each of the diseases, including novel formulations (solid dose, needle free).

- Establish the proof of concept and the mechanism of protection induced by these vaccine candidates

- Provide sufficient data to support the progression of the successful candidates to clinical trial

- Compare and identify different mechanisms by which vaccines induce the desired immune responses

- Confirm the impact of factors such as genes, identified during clinical studies, in the vaccine-induced responses and side effects

Who or what will benefit from these outputs, and how?

The long-term aim is to develop vaccines that will in the future be included in worldwide human vaccination programs. The diseases we are targeting affect primarily babies and young children, and particularly vulnerable populations in developing countries that can benefit most from vaccination (including the elderly). Our ultimate objective is to prevent patients from suffering and dying of these diseases. We expect to discover new vaccines and regimens that will be safe and protect humans from a number of major diseases. Our discoveries, if successful will be tested in human clinical trials and could be included in vaccination programs. In addition, a major expected benefit is the new knowledge that we aim to bring not only into the vaccinology field, but also in the immunology of each of these diseases, through publication of our research.

In addition, our program of work with new vaccines and delivery methods, such as needle-

free parenteral and mucosal delivery, and new technologies such as understanding the genes involved in successful vaccine-induced immune responses may lead to novel knowledge that will significantly and lastingly improve vaccine development programs, efficacy, uptake and safety.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, we follow different strategies:

Publication: we publish the successful as well as the unsuccessful approaches

Conferences: we present our data to the relevant conferences (specific for the diseases we are targeting or conferences focusing on vaccines and vaccine development)

We look out for potential collaborators for all our programs. We have an expertise in vaccine delivery technologies, and how these can be applied to different pathogens. We thus aim to collaborate with experts in the pathogen / disease itself, which allows fruitful collaborations as the expertise and networks are different.

Protection of intellectual property, in order to attract a commercial company to take up the further vaccine development and potentially its commercialisation

Species and numbers of animals expected to be used

- Mice: 9,500

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 do not use animals during vaccine design and production; however, we have to test the vaccines initially in animals before we can trial them in humans to ensure they are safe and efficacious.
Responses to vaccines are complex and at present there is no other way of testing them than using animals, there is no non-animal system that recapitulates the function of the immune system.

The project will use mouse models to evaluate the immunogenicity of vaccines, and also models of human diseases to evaluate if the vaccine can efficiently protect against the diseases.

We use mice because they are the less sentient specie studied, with an immune system that is well characterized, and there is an understanding of how responses in mice may translate into what we would find in humans. There are extensive sets of reagents available for analysing vaccine-induced immune responses in mice. The project also involves genetically altered animals (GAA) in order to investigate the role of specific genes in the immune response, and to identify which part of the immune system provides the immune responses and protection.

The project involves evaluation of the vaccines immunogenicity at ages where the immune system is less reactive (the elderly).

Typically, what will be done to an animal used in your project?

For the immunogenicity studies, mice receive the vaccines by injection twice, and blood samples are taken 5 times to evaluate the response.

For the efficacy studies, mice are immunized twice, blood samples are performed twice prior to challenge. Diseases are induced through experimentally exposing the mice to the bacteria, by injection, or orally, or through spraying the cage with a solution containing the bacteria. Within 2 to 3 days, mice may become ill, and are killed before showing pain and distress, or recover if the pathogen induces a mild disease. For colonization / carriage models, mice don’t become ill.

Very rarely, other explorations of the immune responses may be carried out, involving further injections or using genetically modified mice or aged mice.

What are the expected impacts and/or adverse effects for the animals during your project?

For the immunogenicity studies, the adverse effects expected are mild, these procedures are similar to what would be performed to a human or a baby, except that mice are sedated during the injection to avoid the stress or pain from the manipulation and the injection. Blood samples are performed without sedation, as these are so quick that sedation and short drowsiness would likely induce more stress.

For the challenge studies, mice may become ill and show signs of discomfort (less mobile, loss of body weight). Mice will be monitored daily at the peak of infection and not allowed to suffer discomfort for more than 48 hours. Then they will either recover or be immediately killed. The models of infections are not expected to cause severe pain because the experiments will be stopped before mice become sick, as the effect of the vaccine can be observed by measuring the number of bacteria or virus in the body before it becomes too high.

Expected severity categories and the 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 experiments will be immunogenicity testing, with a mild severity limit (63% of mice or more). For these immunogenicity studies, mice receive the vaccines by injection, and blood samples are taken to evaluate the response. The adverse effects expected are mild, these procedures are similar to what would be performed to a human or a baby, except that mice are sedated during the injection to avoid the stress or pain from the manipulation and the injection. Blood samples are performed without sedation, as these are so quick that sedation and short drowsiness would likely induce more stress.

In up to 21% of mice, we will explore correlates of protection or the mechanism by which the vaccine induces immune responses, and the mice may be subjected to injection of substances other than vaccines. This is not painful, but the increase in amounts of procedures, or old age, increases the stress to the animals, and these studies can thus be

classed in the moderate severity limit.

Finally, up to 16% mice may be subjected to a bacterial challenge, to assess the capacity of the vaccines to protect against the disease. In these studies, mice may become ill and have discomfort for up to 48hours. This is thus a moderate severity limit. The diseases are induced through experimentally exposing the mice to the bacteria or virus. The injections are performed under anaesthetic to avoid pain. Within 2 to 3 days, mice may become ill and show signs of discomfort (less mobile, loss of body weight). Every effort will be made to reduce the welfare cost to these animals by the most refined husbandry methods and providing mashed up food and water on the floor. Mice will be monitored daily at the peak of infection and not allowed to suffer moderate discomfort for more than 48 hours. Then they will either recover or be immediately killed to avoid suffering. The models of infections are not expected to cause severe pain because the experiments will be stopped before mice become sick, as the effect of the vaccine can be observed by measuring the number of bacteria or virus in the body before it becomes too high. Every effort will be made to ensure protocols are continuously refined – in particular by identifying challenge doses and routes of administration that cause reduced animal suffering and distress, and by identifying early timepoints after challenge that allow the evaluation of the vaccine effect without letting mice become ill. Control measures and humane endpoints are used so that any adverse effects experienced by animals are moderately severe at the maximum. All animals will be humanely killed at the end of a specific set of procedures. They will not be kept alive and re-used for other 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?

The program of work involves research on live animals because there is no alternative: the evaluation of the vaccine candidates' immunogenicity and efficacy is the essential part of the research, and there is currently no in vitro system available that can mimic the complexity of the entire immune system. In particular this work proposes to develop vaccine for human use, and thus requires use of a mammal in order to mimic as closely as possible the human immune system. Mice are the standard species used for immunogenicity testing of almost all vaccines. There is a unique body of research on vaccines in mice allowing comparisons with previous work and there are uniquely extensive sets of reagents available for analysing vaccine-induced responses in mice.

Which non-animal alternatives did you consider for use in this project?

There is to date no suitable non-animal alternative to evaluate the immunogenicity of a vaccine. However, several steps of the project benefit from non-animal work:

When a correlate of protection is known for a particular disease (for example the serum bactericidal activity for meningococcal disease), we establish the in vitro assay in house or with collaborators, and routinely use this to avoid the need for challenge experiments in

animal models.

Evaluation of antigen expression in vitro: We quality control and verify the antigen expression or composition / appearance of the vaccines in vitro appropriate techniques, prior to injection into animals: for viral vectors by infection of mammal cells and immunofluorescence or flow-based assays, for outer membrane vesicles by SDS-PAGE and electron microscopy.

Why were they not suitable?

While non-animal alternatives are available to establish some vaccine's potency (such as measuring the amount of antigen), these are not suitable to measure the immune response a vaccine can induce. The in vitro methods do not mimic the complexity of the entire immune system, from antigen presentation, detection and response to the danger signals, migration of immune cells to lymph nodes, generation of germinal centers and generation of antigen-specific B and T-cell 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 number of animals is based on:
the number of projects we have currently 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 project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We aim to follow the ARRIVE guidelines, use statistical power calculations and the NC3R's Experimental Design Assistant to inform 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 together to minimise the number of control mice required: Many experiments necessitate the inclusion of control groups such as unvaccinated animals or control vaccines. To minimise the repeated use of control groups during the project, several test conditions are included simultaneously in each experiment, so that one control group is used as a comparator for many vaccines, rather than assessing one vaccine candidate per experiment which would mean that a control group is required in each experiment.

Sequential sampling: Across a time course, we perform tail-bleeds rather than terminal

bleeds, and have developed protocols allowing the evaluation of the immune responses in small blood volumes. This allows the reduction of the number of animals needed to conduct these studies, as it allows data from multiple time points to be generated from the same group of mice rather than requiring a separate group of animals for each time point. The same approach is used for S. aureus carriage, where simple non-invasive techniques are used (swabs, collection of faeces) to measure the bacterial load, thus allowing long longitudinal studies.

Collaborations: We have established collaboration with groups or companies who have a pre- optimised challenge models to test vaccine efficacy. This allows us to perform challenge experiments without having to use extra mice for optimisation of the model. In addition, these challenge models are well characterized, reproducible and performed by experts, thus allowing a minimum number of animals per group and a minimum stress to the animals.

Pilot studies: when initiating a new protocol, as a challenge study, we perform pilot studies, thus using fewer mice to establish a predictable and reproducible model and then expand its use to assess the scientific question. Similarly, for a new vaccine composition, a short pilot study is performed to assess the reactogenicity and immunogenicity, and if suitable then we expand to more extensive protocols (including comparator groups, long term responses).

Sharing tissues: we collect several organs for assessment of the immune response, and whenever possible provide tissues not relevant to our protocols to colleagues.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of mouse model: Mice have been chosen as they are the standard model species used for initial immunogenicity testing of almost all vaccines.

The project mainly involves the use of wild-type mice (BALB/c, C57BL/6, NIH), including outbred mice (CD-1). Well-characterized strains are used so that the discomfort and sensitivity to challenge agents is already known (from previous publications), for example, BALB/c for S. aureus challenge; sensitive to the challenge but in a highly predictable pattern, allowing a humane end-point to be used prior to severe disease but allowing a robust scientific readout. For enteric bacteria, C56BL/6 and BALB/c are susceptible to challenge and have a predictable pattern of infection, while 129Sv/Ev are resistant and thus allow studies of other aspects of infection.

The project also involves genetically altered animals (GAA) in order to investigate the role of specific genes in the immune response (for example cytokines, TLR receptors, chemokines, T Helper markers). We will use only established GAA strains with known phenotype, so the appropriate care and monitoring can be assigned prior to their arrival.

Mice with immunocompromised phenotype (for example knock-out of a TLR receptor), the risk of infection should be absent due to the housing in IVCs.

We also explore alternatives delivery routes for vaccines that are needle-free: this includes mucosal routes (the vaccine is deposited as a drop in the nose or mouth), and parenteral delivery but without needle (gas-propelled).

We use anaesthesia for some parenteral injections, such as intramuscular injections, as these can be painful.

We also use and refine humane endpoints.

All new staff performing these injections will be trained on cadavers and then animals under anaesthesia.

Increased monitoring is used upon detection of a side effect, and during challenge studies, and pilot studies allow us to have a predictable pattern of side effects.

Refinement of bacterial challenge models: We have use and when possible improve the challenge models described in the literature, including the severity scoring system (used as a scientific readout). We have established challenge models that are reliable, reproducible and inducing low variability.
Close observation and monitoring of the mice allowed us to stop experiments earlier post injections, without compromising the reliability of the model. Only experienced staff monitor the animals during the challenge studies, and we perform pilot studies of any new dose, new strain of microbe or new strain of mice, to identify humane endpoints by close monitoring of the side effects and their timing, before starting experiments in vaccinated animals.

Infectious agents that do not cause systemic illness will be used in preference to pathogen strains that cause systemic illness, where possible, as well as non-invasive exposure routes, for example to establish carriage (by simple contamination of the food and bedding, the bedding and cage is sprayed with a liquid preparation containing the bacteria, the mice become infected simply by nibbling on it or breathing the contaminated air, and this is used instead of handling the mice and applying the bacteria in the nose or mouth). This avoids stress. We also propose to perform administration of antibiotics ip over im route.

We propose to use fasting prior to bacterial challenge by oral gavage: this is an alternative instead of the depletion of gut commensal bacteria with antibiotic treatment prior to enteric infection. Moreover, shortening the fasting period using daytime fasting can be used: a study showed that fasting for 6h gave similar results to fasting for 18 h regarding gastric emptying and intestinal transit time of charcoal (Prior et al., 2009). This is partly due to the fact that mice are nocturnal and eat the major part of their food intake during the night. Therefore, shorter daylight fasting (6 to 10 hours) can reduce the discomfort to the animals. This is an alternative instead of the depletion of gut commensal bacteria with antibiotic treatment prior to Salmonella infection. A milder model of Salmonella infection without antibiotic treatment was developed (Del Bel Belluz et al., PLoS Pathog. 2016 Apr 7;12(4):e1005528), including fasting the animals overnight. Food is provided again after the infection. The course of disease is milder: and thus, is a relevant model to vaccine studies. Controlled access to food prior to infection by oral gavage will be performed only once for that purpose.

When mice are bought aged, request will be made for well-established social groups, so as to induce the least disturbance possible due to transport and new environment.

Why can’t you use animals that are less sentient?

There is a unique body of research on other vaccine types in mice allowing comparisons with previous work, including our own work, which is used to inform our studies. There are extensive sets of reagents available for analysing vaccine-induced immune responses in mice. Although mice have not proven to be perfect predictors of immunogenicity in humans, they continue to provide extremely valuable data, and are thus considered the least-sentient species adequate for this type of work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use the most refined route for the specific vaccine formulations, in keeping with the scientific end point. We use short anaesthesia with isoflurane with most routes when anaesthesia is appropriate (for example not used for oral gavage). The routes and maximum volumes follow the code of practice and guidelines set out in The Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn and Maggie Lloyd, Blackwell publishing, Third Edition (2003). We also explore alternatives delivery routes for vaccines that are needle-free: this includes mucosal routes (the vaccine is deposited as a drop in the nose or mouth), and parenteral delivery but without needle (gas-propelled).

We use anaesthesia for some parenteral injections, such as intramuscular injections, as these can be painful.

We also use and refine humane endpoints.

All new staff performing these injections will be trained on cadavers and then animals under anaesthesia.

Increased monitoring is used upon detection of a side effect, and during challenge studies, and pilot studies allow us to have a predictable pattern of side effects.

Refinement of bacterial challenge models: We have use and when possible improve the challenge models described in the literature, including the severity scoring system (used as a scientific readout). We have established challenge models that are reliable, reproducible and inducing low variability.
Close observation and monitoring of the mice allowed us to stop experiments earlier post injections, without compromising the reliability of the model. Only experienced staff monitor the animals during the challenge studies, and we perform pilot studies of any new dose, new strain of microbe or new strain of mice, to identify humane endpoints by close monitoring of the side effects and their timing, before starting experiments in vaccinated animals.

Infectious agents that do not cause systemic illness will be used in preference to pathogen strains that cause systemic illness, where possible, as well as non-invasive exposure routes, for example to establish carriage (by simple contamination of the food and bedding, the bedding and cage is sprayed with a liquid preparation containing the bacteria, the mice become infected simply by nibbling on it or breathing the contaminated air, and

this is used instead of handling the mice and applying the bacteria in the nose or mouth). This avoids stress. We also propose to perform administration of antibiotics ip over im route.

We propose to use fasting prior to bacterial challenge by oral gavage: this is an alternative instead of the depletion of gut commensal bacteria with antibiotic treatment prior to enteric infection. Moreover, shortening the fasting period using daytime fasting can be used: a study showed that fasting for 6h gave similar results to fasting for 18 h regarding gastric emptying and intestinal transit time of charcoal (Prior et al., 2009). This is partly due to the fact that mice are nocturnal and eat the major part of their food intake during the night. Therefore, shorter daylight fasting (6 to 10 hours) can reduce the discomfort to the animals. This is an alternative instead of the depletion of gut commensal bacteria with antibiotic treatment prior to Salmonella infection. A milder model of Salmonella infection without antibiotic treatment was developed (Del Bel Belluz et al., PLoS Pathog. 2016 Apr 7;12(4):e1005528), including fasting the animals overnight. Food is provided again after the infection. The course of disease is milder: and thus, is a relevant model to vaccine studies. Controlled access to food prior to infection by oral gavage will be performed only once

When mice are bought aged, request will be made for well-established social groups, so as to induce the least disturbance possible due to transport and new environment.

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).

Published literature on challenge m models: regular checks ensure we consider the shortest and mildest possible challenge models.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive the regular NC3R's newsletter. One of our team members is also a member of an animal ethics committee, and this regularly exposed to the improvements made by others working with animals on science. We also keep up with the literature not only specific to the vaccine platforms we are using, but also specific to the diseases and pathogens we are targeting. We have the flexibility to implement these new advances within projects, or when initiating a new one. Most of the times, 3R's advances also improve the workload of experiments, or the reproducibility, and ultimately results in less work, thus benefiting not only the animals but the science too.

214. Antibody Production for Biological and Biomedical Research

Project duration

0 years 6 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Antibodies, Antigens, Peptides, Immunogens

Animal types	Life stages
Mice	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 purpose of this license is to maintain animals started on Project License no 70/8989 for a period of 6 months, whilst a full application for a new License is made.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within 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 production of high quality validated animal derived antibodies are invaluable tools to study biological systems (in all kingdoms) and are vital to identify for example, new biomedical targets for therapeutic benefit. They are also essential for diagnostic kits for swift patient/animal diagnosis

What outputs do you think you will see at the end of this project?

The output will be a product ie the generated animal-derived antibodies. Their production has the potential to yield new basic science information and publications by the end user of the product as well as be used in preclinical and clinical settings to treat disease as well as be used as diagnostics

Who or what will benefit from these outputs, and how?

The animal-derived antibodies will benefit the scientific community by purchase from commercial sources or through availability from the academic research community. The antibodies are critical tools to the biological/chemistry and biomedical sectors for advancing basic research, as well as for therapeutic and diagnostic use.

How will you look to maximise the outputs of this work?

Outputs maximised by constant dialogue with the customer about antigen preparation for the best animal-derived antibody production outcome. Sharing and receiving best practice with relevant stake holders and contributing to researcher outputs and publicising the framework we work.

Species and numbers of animals expected to be used

- Mice: 55
- Rabbits: 37

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, 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 rabbit and mouse will be used. At this life stage the animals have reached maturity that will contribute to a strong immunological response. For maximal outcome success the end-user of the animal-derived antibody will need to use multiple unique target specified antibodies from a range of species and be able to identify them in their application (tissue/diagnostic test). Raising each unique target specified antibody in the same species for use in an individual application makes it impossible to then identify the antibody with a marker tag. The marker tag uses species as its recognition feature. Unique antibodies from the same species will be identified by the same marker tag making it impossible to differentiate between each antibody and specific target. Therefore, access to target specified antibodies from different species allows markers tags recognising each species and thus differentiation between each antibody and specific target.

Typically, what will be done to an animal used in your project?

For the production of an appropriate animal-derived antibody for multiple research purposes, the animals will receive an immunisation with the required antigen/immunogen. After an appropriate interval (2-6 weeks following the primary immunisation) the animals receive "booster" immunisation of the antigen/immunogen. Up to 18 booster immunisation may be administered again every 2-6 weeks. Following this, to obtain polyclonal antibodies, the animal is terminally anaesthetised and bled by cardiac puncture, blood

collected then humanely killed. The collected blood is processed further (by client) to extract the relevant antibodies. For monoclonal antibodies, animals with a strong immune response will receive either an intrasplenic or intravenous antigen/immunogen immunisation. Three- seven days later animals will be humanely killed and the spleen removed again for further processing (by client) to extract the monoclonal antibodies.

What are the expected impacts and/or adverse effects for the animals during your project?

Immediately following immunisation no adverse effects are expected, however initial immunisation may result in weight loss and/or subdued behaviour. There may be discomfort following the injection so pain relief can be administered. These effects should be transient with resolution within 72 hrs. Granuloma (aggregation of white blood cells in response to the procedure) formation may occur at the immunisation site, which should resolve over time (~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)?

Monoclonal antibodies - Moderate -100%
Polyclonal antibodies - 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?

Animals authorised by the previous licence will still be in project when that licence expires. They need to be maintained in order to generate the antibodies.

Which non-animal alternatives did you consider for use in this project?

Replacement technologies exist but the purpose of this PPL is to allow animals still in project at the expiration of the current licence to be maintained while full authorities are applied for.

Why were they not suitable?

The replacement technologies are unsuitable because the purpose of this PPL is to allow animals still in project at the expiration of the current licence to be maintained while full authorities are applied for.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The animals are already in study and need to be maintained while new authorities are sought.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The animals are already in study and need to be maintained while new authorities are sought.

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 are already in study and need to be maintained while new authorities are sought.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods 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 adult life stage for each species (rabbit, mouse) will be used because their immune system is considered to have reached maturity ensuring a complete biological response will be achieved when exposed to an antigen.
The methodology comprises a series of injections of the immunising agent with experience of transient discomfort akin to a human /animal receiving a therapeutic injection eg Covid-19 vaccination.

Why can’t you use animals that are less sentient?

The adult life stage for each species (rabbit, mouse) will be used because their immune system is considered to have reached maturity ensuring a complete biological response will be achieved when exposed to immunising agents.

Animal-derived antibody generation requires an interaction between the immunising agent and the immune system so a living system is required. Adult animals will allow the generation of large volumes of antibody.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All involved staff are already trained to a high standard to carry out the immunisation procedures, welfare and husbandry of the animals. New staff will receive training to maintain the high standards we work to. Good communication among individuals results in quick response and resolution to emerging issues. All these will be maintained. Continuation of the single use needle policy that was introduced in 2019.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3R -https://www.nc3rs.org.uk/experimental-design
NC3R -https://nc3rs.org.uk/reuse-of-needles
NC3R -https://nc3rs.org.uk/grimacescale
LASA Good practice guidelines on Administration of Substance
http://www.verutech.com/pdf/lasa_administration.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Dialogue with colleagues and forum networks undertaking similar work. Engage with NC3R website and webinars (Moving to the Use of animal-free antibodies, July 2020). Within the frame work of relevant advances in relation to the production of animal-derived antibodies, these will be tried alongside existing practices and compared to evolve the best approach to produce high specificity and affinity antibodies.

As already stated novel antibodies still require animal immunisation. Once the novel antibody is produced its sequence can be determined and any future production can take advantage of nonanimal-derived technologies (EURAL ECVAM recommendations, May 2020, EU Directive 2010/63/EU).

215. Investigation into the therapeutic potential of exosomes

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

Exosomes, Therapy, Drug Targeting, Bioengineering, Rare disease

Animal types	Life stages
Mice	adult, juvenile, embryo, neonate, pregnant
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To test the therapeutic potential of a type of naturally occurring molecule secreted by human cells (exosomes). These molecules will be investigated as a novel way to deliver targeted therapies for treatment 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 majority of existing drugs are distributed throughout the body based on their physical and chemical properties. This approach is usually associated with drug reaching its

therapeutic site of action but not always at sufficient concentrations to have a therapeutic effect. Increasing the dose may result in toxicities that limit the amount that can be given. Newer approaches with biological agents including antibodies, cell and gene therapies offer potential to get around this challenge as they are more specific to their target within the body. However, these agents suffer from different challenges, including adverse immunological responses, issues with repeat dosing and challenges around getting the drug to the part of the body where the target is found. We are investigating the potential of exosomes - a type of molecule secreted by the majority of cell types in the human body. In the body these molecules play an important role facilitating different cells communicating with each other. The molecules are found in blood and are administered to humans as a natural component of blood during blood transfusions. They can also be derived from cells in the lab and modified to contain drugs either by encapsulating them or carrying them on their surface (or both). The approach we are taking to produce exosomes in the lab will utilise human cell lines. Because these molecules are derived from human cells, they do not suffer from many of the unwanted properties of other biological drugs, such as eliciting immune responses. To determine how best to apply these molecules to treat human disease, we need to further characterise their properties including where they go to in the body following different dosing routes, whether they can deliver sufficient amounts of drug to the target cells and how long they last.

Once we achieve this, the initial therapeutic area of interest is rare diseases such as argininosuccinic aciduria (ASA), Pompe disease and phenylketonuria (PKU). These diseases either have no treatment or poor treatment options, are often detected very early in childhood life and severely affect lifespan and quality of life.

What outputs do you think you will see at the end of this project?

data to understand the unique properties of different modifications of exosomes and how to apply these to the biggest areas of unmet therapeutic need

data for informing design of preclinical regulatory testing as a precursor to first in man /

clinical trial and potential novel therapeutics

publications to disseminate new findings relating to the use of exosomes as a therapeutic platform

Who or what will benefit from these outputs, and how?

Short term the company will benefit by obtaining data to best position our molecules and enable preclinical regulatory studies and clinical trials.

Medium to longer term it is anticipated that there will be treatment or correction of rare diseases either for which either there are no therapies or where there is significant benefit compared to the current standard of care.

Longer term it is anticipated that treatments will be developed for a broader range of human diseases.

How will you look to maximise the outputs of this work?

Study design will incorporate maximum use of tissues and body fluids (e.g. storage of samples for later studies) from each animal.

Cadaver material not required for the current project may be used by the facility for other researchers / teaching under the establishments tissue sharing policy.

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.

Mice will be used for most experiments because they are the smallest animals with the appropriate physiology for studying potential drug effects. Due to their size they also require less test article. Furthermore, if test articles progress to disease models, the majority of these are genetic models in mice and so this avoids repeating experiments in different species. Rats may be used for studies where the small size of mice makes administration of the test article technically difficult; most likely studies requiring direct dosing into the CNS. Adult animals will be used to enable collection of larger volumes of blood and more tissue for ex vivo analysis, thereby reducing the number of animals needed.

Typically, what will be done to an animal used in your project?

Animals will be administered test article (or control material) either by a single or multiple injections and monitored over time, including blood sampling. Blood will also be collected under terminal anaesthesia and tissues harvested after death. Blood sampling will be based on the guideline volumes provided in the NC3Rs decision tree: https://nc3rs.org.uk/mouse-decision-tree-blood-sampling https://nc3rs.org.uk/rat-decision-tree-blood-sampling

Typically an individual mouse will not provide more than one in-life blood sample, unless the study employs microsampling e.g. for pharmacokinetic studies. Rats will typically provide multiple blood samples via a temporary canula.
Where multiple injections are required, the frequency of dosing will depend on the duration of effect observed following administration of a single dose and the specific route of administration. It is not expected that this would be more regularly than documented in the table for the specific protocol.

What are the expected impacts and/or adverse effects for the animals during your project?

Previous data from our company and other researchers (example publications below), suggests that exosomes themselves have a clean safety profile and therefore adverse effects are limited to pain caused by administering drug or from blood sampling. There is potential for the therapeutic cargo to cause adverse effects but this should be minimal for

therapeutics aimed at the diseases we are investigating as they are largely single protein replacement strategies. Furthermore, it is hypothesised that delivery in exosomes protects animals from potential harmful effects of drugs by delivering them directly to the target.
Escudier B., Dorval T., Chaput N., Andre F., Caby M.P., Novault S., Flament C., Leboulaire C., Borg C., Amigorena S., et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: Results of thefirst phase I clinical trial. J. Transl. Med. 2005;3:10.
Morse M.A., Garst J., Osada T., Khan S., Hobeika A., Clay T.M., Valente N., Shreeniwas R., Sutton M.A., Delcayre A., et al. A phase I study of dexosome immunotherapy in patients with advanced nonsmall cell lung cancer. J. Transl. Med. 2005;3:9.
Besse B., Charrier M., Lapierre V., Dansin E., Lantz O., Planchard D., Le Chevalier T., Livartoski A., Barlesi F., Laplanche A., et al. Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. Oncoimmunology. 2016;5.
Suzuki E., Fujita D., Takahashi M., Oba S., Nishimatsu H. Stem cell-derived exosomes as a therapeutic tool for cardiovascular disease. World J. Stem Cells. 2016;8:297–305.
Dai S., Wei D., Wu Z., Zhou X., Wei X., Huang H., Li G. Phase I clinical trial of autologous ascitesderived exosomes combined with GM-CSF for colorectal cancer. Mol. Ther. 2008;16:782–790.

Expected severity categories and the 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 100% 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?

Animals are needed to understand the properties of the molecules in the whole body, including how long they remain in the blood, where they go within the body and how long they stay in different tissues. They are also needed to reproduce human disease phenotypes to determine pharmacological effect and potential to be developed into a therapeutic.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal models currently available that would address the same questions and provide the necessary data needed for first time in human studies. As we generate data we will explore any in silico methods that become available e.g. for predicting biodistribution. In vitro models including organ on a chip models will continue to be reviewed to determine if these can replace animal use in the future e.g we are currently

investigating in vitro models to assess blood brain barrier penetration. We also have a collaborative project to test pig and human livers that are by products from the meat industry or unsuitable for organ transplant, respectively.

Why were they not suitable?

Currently these models are not validated to the extent that they are ready to replace whole body 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 numbers have been calculated based on the preliminary information on group sizes to give statistical power obtained from contract research organisations. This has then been multiplied by the anticipated number of groups to cover the routes of administration to be tested, targeting strategies, time points and lead candidate therapeutics to be tested. The estimated number of mice also includes the number of mice needed to produce enough homozygous mice necessary for study of disease phenotypes (where heterozygous mice do not display the same phenotype). Statistical support e.g. statistician or use of the NC3Rs Experimental Design Assistant will be utilised for study designs.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Testing as many parameters as possible in vitro to minimise animal testing.
Comparing multiple groups in a single study to avoid duplication (particularly of control animals).

Aiming to miniaturise assays to use less sample and therefore make more measurements from the same animal e.g. for plasma.

We will use the NC3Rs Experimental Design Assistant and statistician support (Ref: https://www.nc3rs.org.uk/experimental-design-assistant-eda). As well as additional statistician support.

Power calculations based on data from externally run studies and collaborators will be used wherever possible.

Reverse translation from clinical studies to ensure minimal in vivo studies to support clinical testing.

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 data from previous and current studies being run at contract research organisations and with collaborators.

Analysing data from single dose studies before planning repeat dose studies.
Tissue and data will be shared across projects within our group or offered to other groups.

Time course studies maximising in life end points to reduce the number of terminal measurements requiring groups at each time point.

Minimising sample volumes needed for analysis (miniaturisation or highly sensitive assay methods) to reduce replication of animals due to restrictions in sampling volumes.

Evaluate newer methodologies as they arise, particularly around in silico predictions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animals used will only undergo procedures to administer therapeutic or control molecules or to sample blood before terminal procedures. Studies will be designed to minimise the volumes of blood needed and will use temporary cannulas where repeat samples are needed with short intervals. Use of a new needle for each animal will be carried out on every study. When testing effective routes of administration, the route with the least pain, suffering and distress will subsequently be used where different routes provide comparable data. The volumes of material injected will be the minimum volume necessary and infusion methods will be considered if larger volumes are needed.
The animals used for these studies will either be healthy animals or animal models selected that best represent the disease phenotype whilst minimising and pain, distress or suffering. Where appropriate, animals with a disease phenotype will be used at younger ages either before symptoms are manifest or when they are milder.

Why can’t you use animals that are less sentient?

The animal models selected are the least sentient available for the project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Indwelling cannulas will be used to replace repeat needle sticks.
Welfare assessments sheets have been devised along with body condition scoring, to monitor animals closely after scientific intervention or surgery.

Technician sheets will accompany all animal studies to define the essence of what procedures have been carried out and the possible observations that could occur directly or indirectly, to ensure optimised daily welfare checks.
Animals undergoing general anaesthesia will always be recovered in a thermostatically controlled warming chamber, to aid recovery.
Analgesia will always be considered with techniques associated with the potential to cause discomfort lasting beyond transient.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult any relevant references listed therein. (Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs.)

We will follow the Good Practise Guidelines as set out by LASA (Reference: https://researchanimaltraining.com/wp-content/uploads/2021/05/lasa_administration.pdf). Animals will be monitored for signs of pain and distress as required by experienced veterinarians and animal care technicians with significant experience in these species. Standard Operating Procedures are employed for animal husbandry and procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs are regularly published on RVC News Forums and other relevant information is circulated by AWERB as relevant to PPL Holders. Whenever possible we will implement these refinements into our studies.